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Guilherme Machado do Carmo

CAMUNDONGOS INFECTADOS EXPERIMENTALMENTE COM *Trypanosoma cruzi*: EFEITO DA DOENÇA DE CHAGAS SOBRE AS ENZIMAS PURINÉRGICAS E TESTES DE UM NOVO PROTOCOLO TRIPANOCIDA

Santa Maria, RS
2018

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação Em Ciências Biológicas: Bioquímica Toxicológica da universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor(a) em Ciências Biológicas: Bioquímica Toxicológica**

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Co-orientadora: Profª. Drª. Daniela Bitencourt Rosa Leal

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Santa Maria, RS

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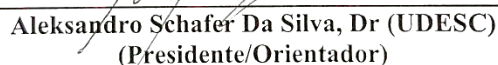

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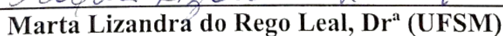

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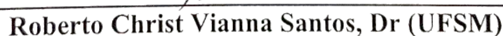

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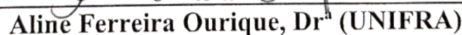

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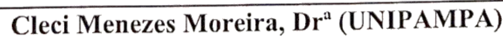

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RESUMO

CAMUNDONGOS INFECTADOS EXPERIMENTALMENTE COM *Trypanosoma cruzi*: EFEITO DA DOENÇA DE CHAGAS SOBRE AS ENZIMAS PURINÉRGICAS E TESTES DE UM NOVO PROTOCOLO TRIPANOCIDA

AUTOR: Guilherme Machado do Carmo
ORIENTADOR: Aleksandro Schafer da Silva

A Doença de Chagas é uma zoonose causada pelo *Trypanosoma cruzi*, presente principalmente na América Latina causando graves problemas de saúde em cerca de 7 milhões de pessoas em regiões endêmicas. Considerando que a Doença de Chagas prossegue ineficaz na cura e que o tratamento possui diversos efeitos colaterais, novas alternativas para o tratamento devem ser avaliadas. Portanto, o objetivo deste estudo foi avaliar a eficácia do tratamento com cordicepina (análogo de adenosina) e pentostatina (inibidor da enzima adenosina desaminase) em camundongos experimentalmente infectados com *T. cruzi* e também verificar se o sistema purinergico é afetado e tem participação na patogenia da Doença de Chagas, visto que, nucleosídeos e nucleotídeos da cascata purinérgica estão envolvidos na fisiopatologia de várias infecções parasitárias. O delineamento do estudo foi dividido em quatro protocolos experimentais. No protocolo experimental I, os animais foram divididos em dois grupos (n=6): grupo controle (não infectadas com *T. cruzi*) e grupo infectado (infectadas com *T. cruzi*) e assim foi investigado o efeito das enzimas purinérgicas em plaquetas, linfócitos e coração de camundongos infectados experimentalmente por *Trypanosoma cruzi* (cepa Y). Como resultado observou-se um aumento na atividade da E-NTPDase (substratos ATP e ADP) e da atividade da E-ADA em linfócitos em camunbdongos infectados com *T. cruzi* (P<0,01). No coração foi observado a presença de infiltrados inflamatórios, assim como múltiplos pseudocistos contendo amastigotas, no entanto a atividade da NTPDase não alterou-se no grupo infectado com *T. cruzi,* porém foi observado uma redução na atividade da 5'-nucleotidase (P<0,001) e um aumento na atividade de ADA nos infectados (P<0,05). A atividade de E-NTPDase (substratos ATP e ADP), E-5'nucleotidase e E-ADA em plaquetas aumentaram significativamente (P<0,05) em camundongos infectados por *T. cruzi*. No protocolo experimental II os animais foram divididos em 10 grupos com seis animais (5 grupos controles e 5 grupos infectados), no qual, os animais dos grupos infectados foram inoculados com 10^4 triposmastigotas da cepa Y. Neste protocolo experimental II foi avaliada a eficácia do tratamento com cordicepina e pentostatina (isolados ou em combinações), bem como o efeito do tratamento sobre as enzimas purinérgicas *in vivo.* A partir desse delineamento constatamos a ineficácia curativa do protocolo experimental, apesar de ter reduzido a parasitemia. Tambem verificamos que as atividades séricas de NTPDase (ATP; P<0,001, ADP; P<0,05) e ADA (P<0,001) foram superiores em camundongos não tratados, mas infectados por *T. cruzi*. No entanto, os camundongos tratados com combinação de 3'-desoxiadenosina e desoxicoformicina foram capazes de modular a atividade de NTPDase (ATP e substrato ADP) e a ADA, impedindo o aumento nos animais infectados (atividade semelhante a animais saudáveis). A atividade da 5'-nucleotidase diminuiu significativamente (P<0,01) nos animais não tratados e infectados, porém o tratamento associado impediu a redução da atividade da 5'-nucleotidase. No protocolo experimental III, os animais foram divididos 5 grupos com seis animais (1grupo controle e 4 grupos infectados), no qual, os animais dos grupos infectados foram inoculados com 10^4 triposmastigotas da cepa resistente ao benzonidazol (cepa Colombiana). Neste protocolo experimental III foi avaliada a eficácia do tratamento com cordicepina e pentostatina (isolados ou em combinações). Neste protocolo também foi realizado testes *in vitro* para avaliar a eficácia dos compostos frente ao *T. cruzi.* Nesse experimento os resultados foram similares ao experimento II, isto é, ineficácia

curativa em camundongos infectados com *T. cruzi*. Já no experimento *in vitro* foi observado uma redução significativa (P <0,001) de epimastigotas e tripomastigotas em grupos tratados com cordicepina e pentostatina (isolada ou combinada), em todas as doses testadas. Para epimastigotas e tripomastigotas a dose letal da associação de cordicepina com pentostatina capaz de matar 50% (DL_{50}) dos parasitas foi de 0,068 mg/mL e 0,027 mg/mL, respectivamente. No protocolo experimental IV, os animais foram divididos em dois grupos (n=6): grupo controle (não infectadas com *T. cruzi*) e grupo infectado (infectadas com *T. cruzi*). Os animais do grupo infectado foram inoculados com 10^4 triposmastigotas da cepa colombiana e foi avaliado o efeito da doença sobre as concentrações séricas de purinas. Neste protocolo observou-se aumento significativo (P <0,05) nos níveis séricos de adenosina trifosfato (ATP), adenosina difosfato (ADP), adenosina (ADO), inosina (INO) e ácido úrico (URIC) em animais infectados. Já os níveis de adenosina monofosfato (AMP) e xantina (XAN) reduziram significativamente (P <0,05). Portando, podemos concluir de modo geral que a infecção por *T. cruzi* altera as enzimas do sitema purinérgico, assim como os níveis de purinas, demonstrando que estão diretamente ligadas com a fisiopatologia da Doença de Chagas. Já o protocolo terapêutico, na dose utilizada, não deve ser recomendado para o tratamento da Doença de Chagas, pois não teve eficácia curativa.

Palavras-chave: Cordicepina, pentostatina, sinalização purinérgica, *T. cruzi*

ABSTRACT

MICE EXPERIMENTALLY-INFECTED WITH *TRYPANOSOMA CRUZI*: EFFECT OF CHAGAS DISEASE ON PURINERGIC ENZYMES, AND EVALUATION OF A NEW TRYPANOCIDAL PROTOCOL

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Chagas disease is a zoonosis caused by *Trypanosoma cruzi*. It occurs primarily in Latin America, where it causes serious health problems for about 7 million people in endemic regions. Considering that Chagas disease currently few effective treatments, and those that exist carry several side effects, new alternatives for treatment should be evaluated. Therefore, the objective of this study was to evaluate the efficacy of cordycepin (an adenosine analogue) and pentostatin (an inhibitor of the enzyme adenosine deaminase) in mice experimentally-infected with *T. cruzi*, and also to verify if the purinergic system is affected and has a role in the pathogenesis of Chagas disease. We did this because nucleosides and nucleotides in the purinergic cascade are involved in the pathophysiology of various parasitic infections. The study design was divided into four experimental protocols. In the experimental protocol I, the animals were divided into two groups (n = 6): control group (not infected with *T. cruzi*), and infected group (infected with *T. cruzi*). We then investigated the effect of purinergic enzymes on platelets, lymphocytes, and hearts in experimentally-infected mice infected with *T. cruzi* (strain Y). We observed increased activity of E-NTPDase (ATP and ADP substrates) and E-ADA in lymphocytes of T. cruzi-infected mice (*P* <.01). We observed inflammatory infiltrates in the heart, as well as multiple pseudocysts containing amastigotes. However, NTPDase activity did not change in the *T. cruzi*-infected group, but there was a reduction in the activity of 5'-nucleotidase (P <.001) and an increase in ADA activity in infected mice (*P* <.05). Activity of E-NTPDase (ATP and ADP substrates), E-5'nucleotidase and E-ADA in platelets increased significantly (*P* <.05) in mice infected with *T. cruzi*. In the experimental protocol II, the animals were divided into 10 groups with six animals (five control groups and five infected groups), in which the animals of the infected groups were inoculated with 104 triposmastigotes of strain Y. In this experimental protocol, we evaluated the efficacy of treatment with cordycepin and pentostatin (isolated or in combination), as well as the effect of treatment on purinergic enzymes *in vivo*. From this design we obtained the curative ineffectiveness of the experimental protocol, despite having reduced parasitemia. We also found that serum NTPDase activity (ATP; *P* <.001, ADP; P <.05) and ADA (*P* <.001) were higher in untreated *T. cruzi*-infected mice. However, mice treated with a combination of 3'-deoxyadenosine and deoxycoformycin were able to modulate NTPDase activity (ATP and ADP substrate) and ADA, preventing the increase in activity in infected animals (i.e., to activity levels similar to those of healthy animals). The activity of 5'-nucleotidase significantly decreased (*P* <.01) in untreated infected animals, but treatment prevented the reduction of 5'-nucleotidase activity. In experimental protocol III, the animals were divided into five groups of six animals each (one control group and four infected groups), in which the animals of the infected groups were inoculated with triphosmastigotes of the benznidazole-resistant strain (Colombian strain). In protocol III, we evaluated the efficacy of the treatment with cordycepin and pentostatin (alone or in combination). In this protocol, we also performed *in vitro* tests to evaluate the efficacy of the compounds against *T. cruzi*. In this experiment, the results were similar to those of protocol II, i.e., ineffectiveness of treatments in mice infected with *T. cruzi*. In the *in vitro* experiment, we observed a significant reduction (*P* <.001) of epimastigotes and trypomastigotes in groups treated with cordycepin and pentostatin (alone or combined) at all doses tested. For epimastigotes and trypomastigotes the lethal doses

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Keywords: cordycepin; pentostatin; purinergic signaling; *T. cruzi*

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LISTA DE ABREVIAÇÕES

Ado - adenosina

ATP- adenosina trifosfato

ADP - adenosina difosfato

AMP - adenosina monofostato

ADA - adenosina desaminase

NTPDases - nucleosídeo trifosfato difosfohidrolases

5´n - 5‘-nucleotidase

P1 - Purinoreceptor tipo 1

P2 - Purinoreceptor tipo 2

IL-2 - interleucina 2

INF-γ - interferon gama

TNF-α - fator de necrose tumoral alfa

HYPO - hipoxantina

XAN - xantina

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1 INTRODUÇÃO

A doença de Chagas é uma antropozoonose causada pelo *Trypanosoma cruzi,* descrita pela primeira vez em 1909 (CHAGAS, 1909). Esta é uma doença negligenciada no mundo e continua a ser um grave problema de saúde pública, com aproximadamente 9-10 milhões de pessoas infectados no mundo (OMS, 2015). Os principais fármacos utilizados para o tratamento da doença de Chagas são o benznidazol e o nifurtimox (COURA & CASTRO, 2002). No Brasil, o benznidazol está disponível para tratar pacientes com aproximadamente 80% de efetividade na fase aguda da doença e <20% dos pacientes com doença de Chagas crônica (COURA & CASTRO, 2002; CHATELAIN, 2015). Após a infecção por *T. cruzi* o parasito induz alguns processos patológicos nos vertebrados, dentre eles podemos citar a resposta inflamatória, resposta imunológica. A sinalização purinérgica desempenha um importante papel na modulação da resposta imunológica e inflamatória por purinas extracelulares.

O sistema purinérgico é conhecido por ser uma via de sinalização importante em diversos tecidos, desencadeando múltiplos efeitos celulares, tais como: a resposta imune e a inflamatória, a dor, a agregação plaquetária, a vasodilatação mediada pelo endotélio, a proliferação e a morte celular (BURNSTOCK, 2006). Três componentes principais fazem parte do sistema purinérgico: nucleotídeos e nucleosídeos extracelulares, seus receptores e ectoenzimas responsáveis pela regulação de níveis destas moléculas (YEGUTKIN, 2008). Níveis elevados de adenosina trifosfato (ATP) pode interagir com receptores P2, desenvolvendo um perfil pró-inflamatório em células imunes (BOURS et al., 2006). Além disso, o ATP e a adenosina difosfato (ADP) induzem efeitos cardiovasculares, tais como vasodilatação, aumento da frequência cardíaca e agregação de plaquetas (DE VENTE et al., 1984; AGCHESCH et al., 1999). No entanto, o nucleosídeo adenosina age como um composto anti-inflamatório nas células e pode proteger o coração de algumas doenças (BOURS et al., 2006; ROBSON et al., 2006).

O tratamento convencional da Doença de Chagas não é 100% eficaz e ainda possui efeitos colaterais (OLIVEIRA et al., 2015). Sabendo que o tratamento com cordicepina (análago da adenosina) combinado com pentostatina (inibidor da adenosina desaminase) age na via metabólica dos tripanosomas (ROTTENBERG et al., 2005) e que já apresentou eficácia frente a tripanossomas (WILLIAMSON & MACADAM, 1976; ROTTENBERG et al. 2005; DALLA ROSA et al., 2013). O objetivo deste estudo foi avaliar se as enzimas do sitema purinérgico de camundongos infectados experimentalmente com *Trypanosoma cruzi* é afetada e tem participação na patogenia da doença, tendo em vista que os nucleosídeos e nucleotídeos

da cascata purinérgica estão envolvidos na fisiopatologia de diversas infecções parasitárias, assim como testar a eficácia de um novo protocolo terapêutico da Doença de Chagas experimental, usando um análogo de adenosina (cordicepina) e um inibidor da enzima adenosina desaminase (pentostatina).

2 REVISÃO DE LITERATURA

A Doença de Chagas ou Tripanosomíase americana é uma zoonose causada pelo *Trypanosoma cruzi* (CHAGAS, 1909), presente principalmente na América Latina. É um sério problema de saúde pública por afetar cerca de 6-7 milhões de pessoas no mundo (BERN, 2015). Além disso, mais de 25 milhões de pessoas estão em risco de contrair a Doença de Chagas (OMS, 2015). De acordo com a Organização Mundial da Saúde (OMS, 2015), o Brasil é um dos países com maior ocorrência de Doenças Tropicais Negligenciadas, dentre elas a Doença de Chagas, que até o ano de 2010 afetava cerca de um milhão e novecentas (RASSI-JÚNIOR et al., 2010).

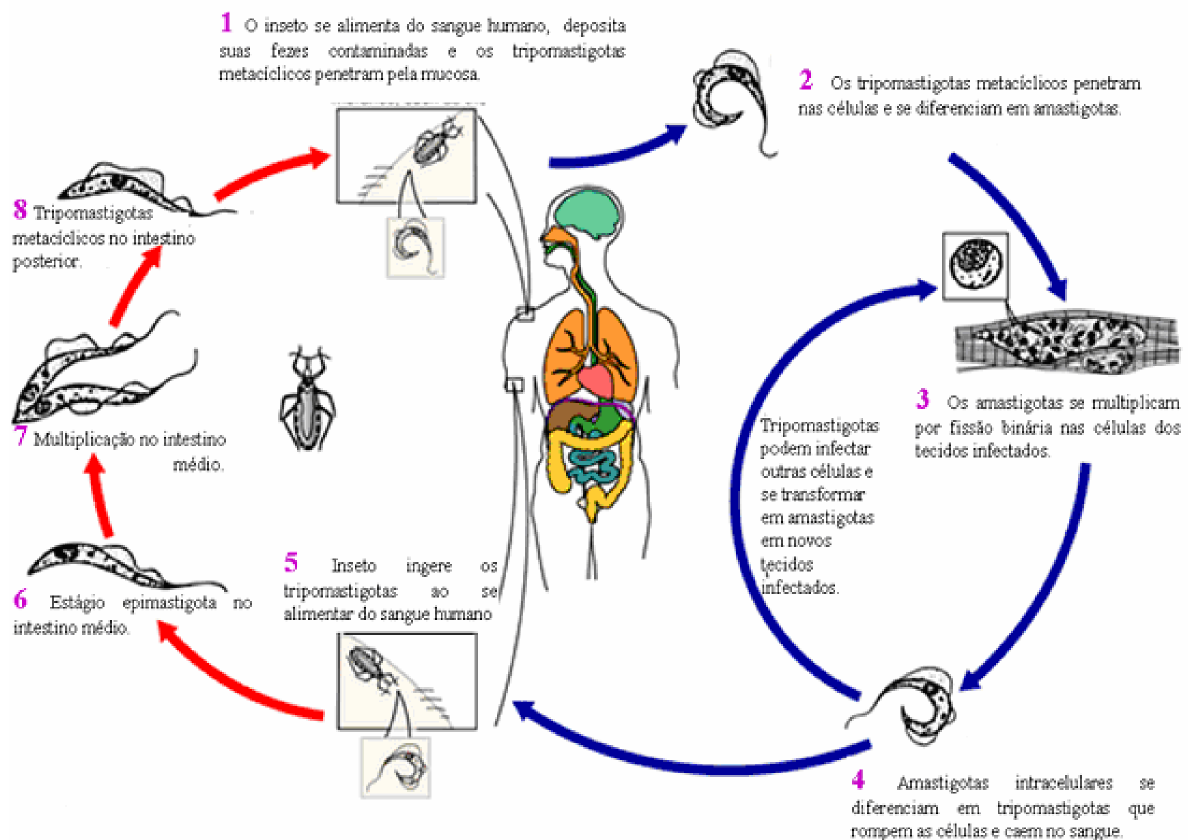
O agente etiológico da Doença de Chagas é o protozoário flagelado pertencente da Ordem Kinetoplastida e da Família Trypanosomatidae chamado de *Trypanosoma cruzi* (NETO e PASTERNAK, 2009; CLAITON, 2010). O parasito foi descoberto em 1909, pelo cientista brasileiro Carlos Justiniano Ribeiro Chagas (CHAGAS, 1909). No entanto, a doença de Chagas é provavelmente uma doença antiga, pois o DNA de *T. cruzi* foi registrado em espécimes de tecido de múmias em países andinos pré-colombianos à 9000 anos (AUFDERHEIDE et al., 2004). Este protozoário possui um ciclo evolutivo complexo e compreende diferentes estágios de desenvolvimento, entre suas formas destacamos a forma epimastigota e tripomastigota metacíclica infectante, presentes no inseto vetor e as formas amastigotas e tripomastigotas presentes no hospedeiro vertebrado (TANOWITZ et al., 1992).

O ciclo biológico (Figura 1) do parasito é do tipo heteroxênico e passa por diferentes formas evolutivas no interior do hospedeiro e nos insetos vetores. Os vetores da Doença de Chagas são insetos da ordem Hemiptera, subfamília Triatominae e conhecidos popularmente no Brasil como “barbeiro”. Os insetos com hábitos geralmente noturnos são infectados quando se alimentam de sangue de um animal ou humano infectado, assim as tripomastigotas no lúmen do intestino transformam-se em epimastigotas. Estas se multiplicam no tubo digestivo do inseto e diferenciam-se na porção terminal em tripomastigotas metacíclicas. Estes insetos infectados ao se alimentarem do sangue do hospedeiro vertebrado defecam nas proximidades da picada, podendo então transmitir através da pele lesada ou mucosa as formas tripomastigotas metacilícas infectantes, fechando assim seu ciclo de vida (BRASIL, 2009; MARTINS et al., 2012). Estas formas tripomastigotas metacíclicas quando penetram no local da picada invadem diversos tipos de tecidos e realizam seu ciclo intracelular, onde se diferenciam na forma

amastigota. Após um período de três a quatro dias, os amastigotas se diferenciam em tripomastigotas, que são liberados para a circulação sanguínea iniciando o próximo ciclo de infecção em outras células (BRENER, 2000; COURA e DIAS, 2009).

O homem passou a fazer parte do ciclo de transmissão, quando invadiu o ecótopo natural compreendido pelos vetores e seus hospedeiros mamíferos. Dessa forma, os vetores passaram a co-habitar as instalações humanas colonizando os mais variados locais como buracos ou fendas em paredes, atrás de quadros ou pintura solta. Nota-se maior incidência em casas de condições precárias como as de pau a pique, sapé, madeira ou barro, sendo mais frequente, nas zonas rurais e entre a população de baixa renda (Rodríguez-Bonfante et al., 2007).

Figura 1 Ciclo biológico do *Trypanosoma cruzi* (Adapatado de Fonte CDC, 2017)



A transmissão da Doença de Chagas pela via vetorial é considerada o mecanismo de transmissão de maior relevância epidemiológica. No entanto, a doença pode ser transmitida por transfusão sanguínea, transmissão congênita, transplante de órgãos e acidentes laboratoriais (RASSI-JÚNIOR et al., 2012; OMS, 2015). O parasito só é transmitido de pessoa a pessoa por meio de sangue, transplante de órgãos e via placenta, sendo que a maioria dos indivíduos infectados irá carregar a doença por toda vida, sendo assim esses pacientes são impedidos de doações de sangue e órgãos (BRASIL, 2009). Também é observado contaminação através do consumo de alimentos infectados com fezes de triatomíneo. Registros mostram infecção de Doença de Chagas devido à ingestão do fruto do açaí e de caldo de cana contaminados pelo *T. cruzi* (STEINDEL et al., 2008). Assim, o curso da infecção nos vertebrados suscetíveis é influenciado por fatores como a idade, sexo, constituição genética do hospedeiro, características genéticas, morfológicas e biológicas da cepa infectante e fatores externos como temperatura ambiental (Brener et al., 2000).

A infecção humana por *T. cruzi* possui basicamente duas fases, isto é, uma aguda e outra crônica (MONCAYO e SILVEIRA, 2009). A fase aguda caracteriza-se pela presença do parasito circulante na corrente sanguínea em alta quantidade, podendo persistir por até 4 meses. Durante essa fase pode haver quadros febril e sinal de Romanã, desaparecendo espontaneamente os sinais e sintomas clínicos, e assim evoluindo para a fase crônica, ou progredindo para formas agudas graves, que podem levar ao óbito (PRATA, 2001). Já na fase crônica, os parasitos circulantes na corrente sanguínea desaparecem e pode apresentar-se como indeterminada, neurodegenerativa, cardíaca, digestiva ou cardiodigestiva. As complicações cardíacas são as alterações mais graves e frequentes, sendo que acometem 20 a 40% dos pacientes após a infecção (HIGUCHI et al., 2003; RASSI-JÚNIOR et al., 2009).

O *T. cruzi* é uma população heterogênea, composta por um grupo de cepas que apresentam origem e características diferentes, como epidemiologia, patogênese, resposta ao tratamento, entre outras. As populações de *T. cruzi* são agrupadas em biodemas conforme padrões de comportamentos semelhantes, tais como: picos de parasitemia, morfologia das formas, tropismo tecidual, taxa de mortalidade, predomínio das formas largas ou delgadas e lesões histopatológicas (Andrade & Magalhães,1997). O biodema I é caracterizado por cepas que se multiplicam rapidamente, com alta parasitemia e mortalidade entre 7 e 12 dias pós infecção, como representante temos a cepa Y. As cepas do biodema II são caracterizadas por ter uma multiplicação lenta, com picos de parasitemia entre 12 e 20 dias pós infecção, com alta mortalidade neste período, como representante deste temos a cepa São Felipe. A cepa colombiana, resistente ao benzonidazol (NEAL & BUEREN, 1988) possui multiplicação lenta,

com picos de parasitemia após 20 dias de infecção e é representante do biodema III (DEVERA et al., 2003). Esta variabilidade genética pode contribuir por diferentes manifestações clínicas e mortalidade da Doença de Chagas.

A Doença de Chagas pode ser tratada com benzonidazol e nifurtimox (COURA e CASTRO, 2002), sendo o benzonidazol um fármaco específico para o tratamento, já o nifurtimox é utilizado quando ocorre intolerância ao primeiro fármaco (CASTRO et al., 2006). Ambos os medicamentos são "quase" 100% eficaz na cura da doença, se administrada logo após a infecção, no início da fase aguda. Estes elimininam as formas tripomastigotas sanguíneas inibindo a síntese de proteínas e de RNA do parasito (GONZALEZ & Cazzulo, 1989). No entanto, na fase crônica as chances de cura são menores que 20% (FERREIRA, 1990; CHATELAIN, 2015). O tratamento também é indicado para aqueles em que a infecção foi reativada (devido à imunossupressão), para crianças com infecção congênita, e para os pacientes durante a fase crônica precoce (OLIVEIRA et al., 2008).

Basicamente são três os processos patológicos básicos que o parasito induz nos vertebrados: a resposta inflamatória, as lesões celulares e a fibrose. Estes processos podem ocorrer em diversos órgãos e tecidos em humanos, aparecendo com maior frequência no coração, tubo digestivo e sistema nervoso (COURA e BORGES-PEREIRA, 2010). Após a infecção em humanos por agentes patogênicos, respostas imunes são montadas para controlar a infecção, mas para isso, diferentes sistemas inflamatórios são ativados, muitos deles pouco conhecidos. Entre esses destacamos o sistema de sinalização purinérgica que desempenha um importante papel na modulação das respostas inflamatórias e imunes por purinas extracelulares tais como adenosina trifosfato (ATP) e adenosina (ADO) (ZIMMERMANN, 2001; YEGUTKIN, 2008).

Sistema purinérgico (Figura 2), é caracterizado por ser uma importante via de sinalização em diversos tecidos, desencadenado multiplos efeitos, tais como resposta imunológica, inflamação, dor, agregação plaquetária, vaosdilatação (JUNGER, 2011). Estes sinalizadores moleculares e suas concentrações são controlados pela expressão e atividade de enzimas que metabolizam tais moléculas (ZIMMERMANN, 2000). Diferentes tipos celulares, como células endoteliais, plaquetas, macrófagos, eritrócitos, linfócitos, entre outras células, expressam componentes da sinalização purinérgica (ZIMMERMANN et al., 2012).

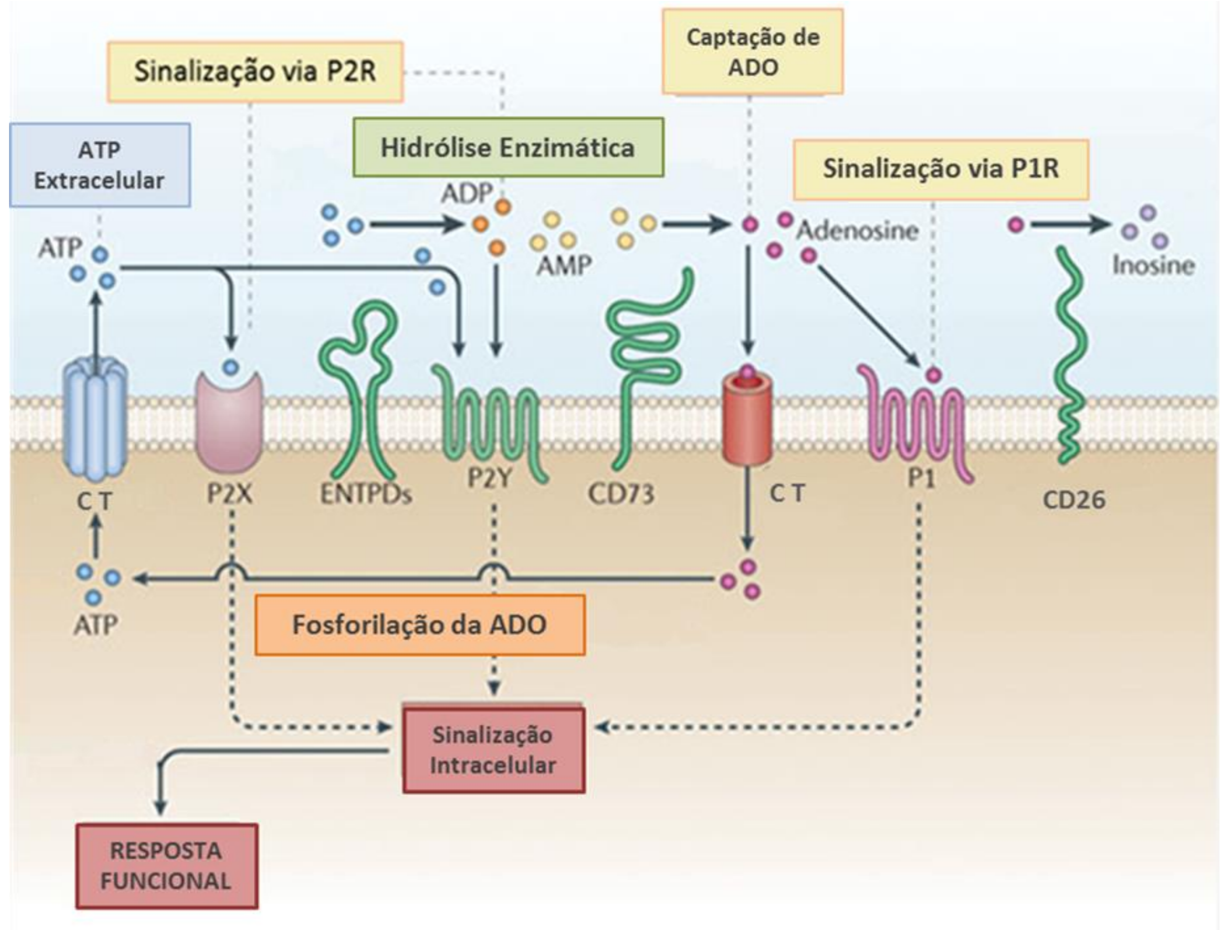
A sinalização purinérgica é composto por nucleotídeos (ATP e ADP) e e nucleosídeos (adenosina) extracelulares que são responsáveis por mediar a sinalização; receptores purinérgicos específicos (P2X, P2Y e P1); e as ectoenzimas que são responsáveis pelo controle dos níveis de mediadores no meio extracelular, entre estas enzimas destacamos a nucleosídeo

trifosfato difosfohidrolases (NTPDases), 5‘-nucleotidase (5’n) e a adenosina desaminase (ADA) (JUNGER, 2011)

O nucleotídeo ATP liberado extracelularmente participa de inúmeras funções biológicas, tais como a contração muscular, neurotransmissão, agragação plaquetária e em processos inflamatórios (BOURS et al., 2006, p. 358). O ATP liberado no meio extracelular devido a dano celular, estimulo por patógenos, estresse oxidativo é interpretado pelo sistema imunológico como um “sinal de perigo” e participa de diversos processos para a instalação de uma resposta inflamatória (JUNGER, 2011). Este nucleotídeo interage com receptores do tipo P2X e P2Y de linfócitos e outras células imunes e estimula a ativação, proliferação e migração celular para focos inflamatórios (BOURS et al., 2006). Também induz a secreção de citocinas pró-inflamatórias, como a interleucina 2 (IL-2), interferon gama (INF-γ), fator de necrose tumoral alfa (TNF-α), entre outras (BOURS et al., 2006). O nucleotídeo ADP é o primeiro produto da hidrólise do ATP, sendo conhecido por interagir apenas com receptores P2Y, sendo um importante agregador plaquetário endógeno, apresentando também ação vasoconstritora (BOURS et al., 2006). Em situação de dano vascular o ADP é liberado no interior de grânulos existentes nas plaquetas, sendo assim, considerado um nucleotídeo importante para o recrutamento plaquetário e indutor de formação de trombos no interior de vasos sanguíneos (PARK & HOURANI, 1999). O nucleotídeo adenosina monofosfato (AMP) é um metabólito intermediário do ATP. Este não interage com nenhum receptor purinérgico, no entando, a degradação enzimática deste nucleotídeo é responsável pela formação de adenosina no meio extracelular, sendo assim importante para a regulação da sinalização dos receptores P1 (BOROWIEC et al., 2006). A adenosina desempenha um importante papel como agente anti-inflamatório endógeno quando interage com os receptores P1 induzindo a supressão de linfócitos (CROSTEIN, 1994) e reduzindo a liberação de citocinas pró-inflamnatórias (DOS REIS et al., 1986). A adenosina também é responsável por inibir a agregação plaquetária promovida pelo ADP, e capaz de induzir a vasodilatação, além de reduzir a frequência cardíaca (BIRK et al., 2002; BOURS et al., 2006).

Os níveis destes nucleotídeos extracelulares são controlados por nucleotidases que são enzimas solúveis ao longo de tecidos intersticiais ou ancoradas na membrana plasmática das células. A enzima NTPDase (EC 3.6.1.5) ou E-NTPDase (CD39), é responsável pela hidrólise do ATP em ADP e pela hidrolise do ADP em adenosina monofosfato (AMP). O nucleotídeo AMP é hidrolisado por 5'-nucleotidase (EC 3.1.3.5) em adenosina, que é submetida a desaminação em inosina pela enzima adenosina desaminase (ADA, EC 3.5.4.4) ou E-ADA quando ancorada na membrana de células (ZIMMERMANN, 2001; COLGAN et al., 2006).

Figura 2 Visão geral do sistema purinérgico. Adaptado de Junger (2011)



De acordo com Burnstock et al. (2009), nucleosídeos e nucleotídeos são responsáveis por uma variedade de funções biológicas em curto prazo e/ou a longo prazo de sinalização, incluindo a inflamação. Estas sinalizações são mediadas pela família ecto-nucleotidase, que são responsáveis pelo controle dos níveis desses nucleotídeos e nucleosídeos conforme mencionado no paragrafo anterior. No entanto, a cascata purinergica continua com a inosina sendo hidrolisada em hipoxantina (HYPO), que são hidrolisadas em xantina (XAN). Por último, o XAN é hidrolisado em ácido úrico. É importante enfatizar que esses nucleosídeos e nucleotídeos estão envolvidos na fisiopatologia de várias infecções parasitárias (BALDISSERA et al., 2016; DOLESKI et al., 2016), incluindo outras espécies de *Trypanosoma* spp (DA SILVA et al., 2012).

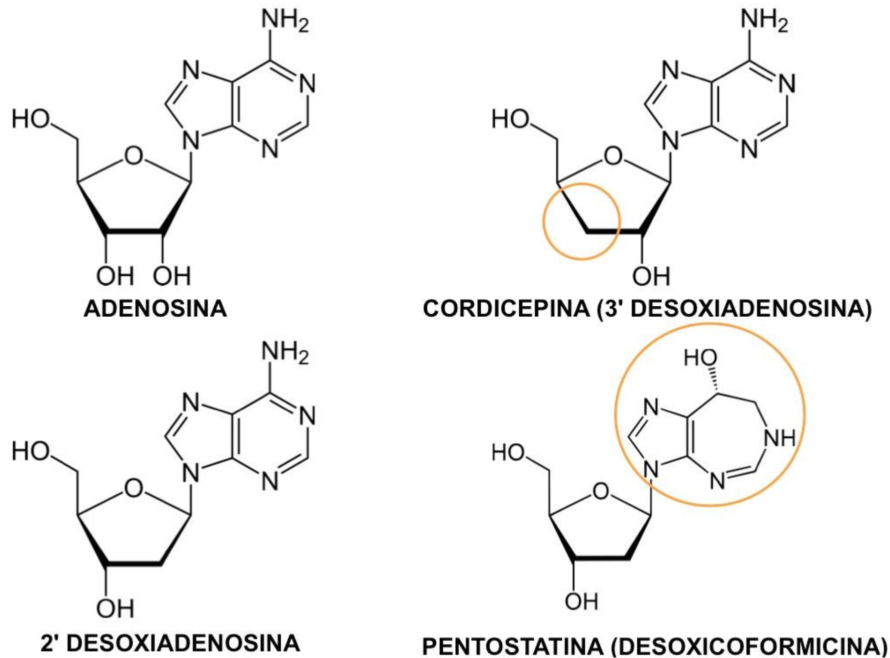
Conforme já mencionado, o tratamento contra a Doença de Chagas é baseado em nifurtimox e benznidazol, porém efeitos colaterais são relatados em humanos com o uso destes princípios ativos. Entre os efeitos colaterais destaca-se a anorexia, dor abdominal, náuseas, vômitos e perda de peso, dermatite, anorexia, anemia e leucopenia (OLIVEIRA et al., 2015). Um novo protocolo usado em outros tripanossomas tem apresentado sucesso terapêutico, assim como cura de animais experimentalmente infectados, sendo este a base de um análogo de adenosina e inibidor da adenosina deaminase (ADA).

A cordicepina (3'-Deoxiadenosina) (Figura 3) é isolada do fungo *Cordyceps miltaris* e tem sido proposto como ativo na medicina tradicional chinesa devido as numerosas atividades biológicas reportadas, tais como: indução do apoptose (CHEN et al., 2014), inibição da agregação plaquetária (CHO et al., 2006), inibição da inflamação (ZHANG et al., 2014), anti-tumoral (JEONG et al., 2012; LU et al., 2014). O potencial tripanocida da cordicepina (análogo da adenosina) foi observado por Williamson e Macadam (1976), Rottenberg et al. (2005) e por Vodnala et al. (2008) devido à incapacidade dos tripanossomas em sintetizar novas purinas e a consequente dependência destes nucleosídeos dos fluidos corporais de seus hospedeiros. No entanto a administração somente da cordicepina não resultou em cura completa da infecção por *Trypanosoma vivax* e *Trypanosoma evansi* (AIYEDUN et al, 1973; DA SILVA et al., 2011). Por outro lado, estudos (ROTTENBERG et al., 2005; VODNALA et al., 2008), mostraram que a cordicepina combinada com pentastotina (Deoxicoformicina) (figura 3), um inibidor da ADA, teve efeito curativo na infecção por *T. brucei* em camundongos, na fase aguda e crônica da doença.

Assim, foi constatado que a cordicepina e a pentostatina agem na via metabólica dos tripanosomas de uma maneira que os fármacos atualmente disponíveis não fazem (ROTTENBERG et al., 2005). Naturalmente o organismo do hospedeiro produz a adenosina e a desoxiadenosina, as quais são moléculas vitais aos tripanossomas. Devido a grande semelhança com cordicepina e pentostatina, algumas enzimas de reconhecimento da adenosina e da desoxiadenosina acabam confundindo-as (TSAI, et al., 2010). Quando a cordicepina, é administrada em um hospedeiro infectado por tripanossomas, ela é confundida pela adenosina kinase e é fosforilada a AMP, ADP e ATP e assim inserida na molécula de RNA. Após ser inserida, a síntese irá parar, pois a cordicepina não possui a hidroxila no carbono 3’ onde o grupo fosfato se ligaria (SIEV, et al., 1969). A penstostatina, um análogo da desoxiadenosina, quando em contato com o hospedeiro infectado por tripanossomas ocorrerá erro nas enzimas que fosforilam a desoxiadeanosina, e assim as enzimas que inserrem o dATP na síntese de DNA irão inserir a pentostatina. Portanto, haverá uma falha na geração da fita dupla de DNA, pois a pentostatina possui uma base

diferente da base adenina encontrada na desoxiadenosina. Além disso, a pentostatina é um inibidor irreversível da ADA, e o bloqueio da ADA leva ao acumulo e proteção contra a degradação da cordicepina, fazendo com que ela desenvolva a sua ação (SPIERS, 1987).

Figura 3 Moléculas de adenosina e desoxiadenosina com seus respectivos análogos, cordicepina e pentostatina (Dalla Rosa, 2014).



Considerando que a Doença de Chagas prossegue ineficaz na cura e que o tratamento possui diversos efeitos colaterais, novas alternativas para o tratamento devem ser avaliadas. Dalla Rosa et al. (2013) sugerem uma eficácia curativa da combinação de cordicepina e pentostatina em camundongos infectados experimentalmente com *T. evansi.* Neste contexto, surge a hipótese era que esse tratamento também tenha efeito positivo para *T. cruzi,* sendo uma opção terapêutica para Doença de Chagas.

3. OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar se as enzimas do sitema purinérgico de camundongos infectados experimentalmente com *Trypanosoma cruzi* é afetada e tem participação na patogenia da doença, assim como testar a eficácia de um novo protocolo terapêutico da Doença de Chagas experimental, usando um análogo de adenosina (cordicepina) e um inibidor da enzima adenosina desaminase (pentostatina).

3.2 OBJETIVOS ESPECÍFICOS

Investigar se doença causa alterações nas enzimas purinérgicas em plaquetas, linfócitos e coração de camundongos infectados experimentalmente com *Trypanosoma cruzi* (cepa Y), participando a resposta inflamatória e hemostasia.

Verificar se o tratamento alternativo a base de cordicepina e pentostatina em camundongos infectados experimentalmente com *Trypanosoma cruzi* (cepa Y e cepa Colombiana) é eficaz no controle e cura da doença.

Avaliar se o tratamento alternativo influencia alterações nas enzimas do sistema purinérgico em camundongos infectados experimentalmente com *Trypanosoma cruzi* com a cepa padrão Y.

Avaliar se a Doença de Chagas influencia alterações nos níveis séricos de purinas em camundongos infectados experimentalmente com *Trypanosoma cruzi* com a cepa colombiana.

4. RESULTADOS

O projeto foi aprovado pelo CEUA da UFSM (protocolo 8288290615), conforme consta no Anexo 1.

Os resultados desta Tese estão apresentados na forma de três artigos científicos publicados e um artigo aceito para publicação em revistas internacionais (Anexo 2, 3, 4 e 5), assim como um manuscrito em fase de revisão em revista cientifica.

4.1. ARTIGO I

Publicado na revista: **Experimental Parasitology**

Purinergic enzymatic activities in lymphocytes and cardiomyocytes of mice acutely infected by *Trypanosoma cruzi* modulating the inflammatory responses

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Abstract

The aim of this study was to evaluate the activity of purinergic enzymes in lymphocytes and cardiac tissue of mice experimentally infected by *Trypanosoma cruzi*. Twelve female mice were used, divided into two groups (n=6): uninfected and infected. On day 12 post-infection (PI), the animals were anesthetized and after euthanized, and samples were collected for analyses. Infected mice showed reduction in erythrocyte counts, hematocrit and hemoglobin concentration, as well as reduced number of total leukocytes in consequence of neutropenia ($P<0.01$). The number of monocytes increased in infected mice ($P<0.001$), however the number of lymphocytes and eosinophils did not differ between groups ($P>0.05$). The E-NTPDase (ATP and ADP substrate) and E-ADA activities in lymphocytes increased significantly in mice infected by *T. cruzi* ($P<0.01$). In the heart, multiple pseudocysts containing amastigotes within cardiomyocytes were observed, as well as focally extensive severe necrosis associated with diffuse moderate to severe inflammatory infiltrate of lymphocytes. Although, the NTPDase activity (ATP and ADP substrate) in the cardiac homogenate did not differ between groups, a reduction on 5'-nucleotidase activity ($P<0.001$) and an increase in the ADA activity in infected animals ($P<0.05$) were observed. Thus, animals infected by *T. cruzi* experienced the disease, i.e., showed anemia, leucopenia, and heart lesions. Associated with this, purinergic enzymes showed altered activities, which might be related to the modulation of the inflammatory response.

Keywords: Chagas disease; inflammatory response; NTPDase, 5’-nucleotidase, ADA.

INTRODUCTION

Chagas disease is a zoonosis caused by *Trypanosoma cruzi* (Chagas, 1909), present mainly in Latin America and remains a serious health problem affecting about 6–7 million people in the American continent. Also, more than 25 million people are at risk of contracting Chagas disease in the world (WHO, 2015). This disease is featured for two phases: acute and chronic. The acute phase, which appears after one week of *T. cruzi* infection, causes low (<10%) mortality. After 1-2 months of infection, the immune system partially controls infection that persists for all life. Approximately 60 to 70% infected untreated people never develop clinical manifestations (indeterminate phase). However, the remaining 30 to 40% develop symptomatic chronic phase of the disease 10 to 30 years after infection, characterized by cardiomyopathy, megacolon, and megaesophagus (Chatelain, 2015; Steverding, 2014; Teixeira et al., 2011). These pathological changes might be due to different mechanisms and may affect other systems, such as the purinergic system that is involved in many physiopathological events in several acute diseases (Castilhos et al., 2015; Oliveira et al., 2012; Tonin et al., 2013), as investigated in this study.

The purinergic signaling system plays an important role in modulating the inflammatory and immune responses by extracellular purines such as ATP and adenosine (Yegutkin, 2008). High ATP levels can interact with P2 receptors, developing a pro-inflammatory profile in immune cells (Bours et al., 2006). Moreover, ATP and ADP induce cardiovascular effects, such as vasodilation, increased heart rate, and platelets aggregation (Agteresch et al., 1999; De Vente et al., 1984). However, the nucleoside adenosine (Ado) acts like an anti-inflammatory compound in cells, and can protect the heart from some diseases (Bours et al., 2006; Cronstein et al., 1994). The levels of these extracellular nucleotides are controlled by nucleotidases that are soluble enzymes throughout interstitial tissues or anchored in the plasma membrane of cells. The enzyme NTPDase (EC 3.6.1.5) or E-NTPDase (CD39), when anchored in lymphocytes, are responsible for the hydrolysis of ATP and ADP, into AMP. The nucleotide AMP is hydrolyzed by 5′-nucleotidase (EC 3.1.3.5) into adenosine, and adenosine undergoes deamination into inosine by adenosine deaminase (ADA; EC 3.5.4.4) or E-ADA when anchored in lymphocytes (Colgan et al., 2006; Zimmermann, 2001). Several studies are revealing the physiological importance of the nucleotidases in the heart during parasitic diseases (Robson et al., 2006). Considering that, *T. cruzi* infection induces systemic inflammation and cardiac pathophysiological changes, the aim of this study is to investigate the activities of the

nucleotidases and E-ADA in lymphocytes and cardiac tissue of mice experimentally infected by *T. cruzi*.

MATERIALS AND METHODS

Chemicals

The substrates ATP, ADP, AMP and adenosine, as well as Trizma base, Coomassie Brilliant Blue G and bovine serum albumin were obtained from Sigma Chemical Co (St. Louis, MO, USA) and K_2HPO_4 from Reagen. All chemicals used in this experiment were of high purity.

Strain Y

This study used blood trypomastigotes of *T. cruzi* known as strain Y (Silva & Nussenweig, 1953) cryopreserved in liquid nitrogen. This strain causes acute disease in laboratory animals.

Animal model and experimental design

Twelve female (Swiss) mice (45 days of age, 20 – 30 g of body weight) from the Central Animal House of the Universidade Federal de Santa Maria (UFSM) were used in this experiment. They were maintained in a room with constant temperature (23 ± 1 ºC) on a 12 h light/dark cycle with free access to feed and water. They were divided into two groups: uninfected (the control group), and infected group with six animals each. Animals of the infected group were intraperitoneally inoculated with blood containing 10^4 trypomastigotes of *T. cruzi* (strain Y) from a mice previously infected.

Blood parasitemia evaluation

The infection was monitored by counting the number of motile parasites in 5 µL of fresh blood sample drawn from the lateral tail vein, as recommended by a standard protocol (Brener 1962). The number of blood trypomastigotes was recorded in a two-day interval from 2 to 12 days post-infection (PI), and the number of parasites was expressed as parasites/mL of blood.

Sample collection

Twelve days PI, the animals were anesthetized with isofluorane and humanely euthanized by decapitation. Blood samples were stored in tubes containing 7.2 mg of

dipotassium EDTA anticoagulant for hematological analyses, and tubes containing citrate as anticoagulant for isolation of lymphocytes. Thereafter, their heart were removed and a portion weighed and homogenized with Tris–HCl 50 mM and 4 mM EDTA (to exclude possible interference of endogenous divalent cations). Each homogenate was centrifuged at 2200 g for 10 min with the supernatant collected and frozen at −20 °C until analyses.

Hematological analyses

Hematological parameters were assessed in whole blood collected in tubes containing EDTA (Vacutainer®) using an automatic counter COULTER T890® (Coulter Electronics, Inc, Hialeach, FL, USA). Total leukocytes (WBC), total erythrocytes (RBC), hematocrit (Ht), and hemoglobin concentration (Hb) were measured. Blood smears were fixed in methanol and stained with Instant-Prov (NewProv®) stain for the determination of differential WBC counts where at least 200 WBCs counts were performed.

Isolation of lymphocytes

Lymphocytes-rich mononuclear cells were isolated from peripheral blood collected with 129 mM sodium citrate as anticoagulant and separated on Ficoll-Histopaque density gradient, as described by Böyum (1968). Protein in lymphocytes was measured by the Comassie Blue method according to Bradford (1976), using serum albumin as standard.

E-NTPDase, and 5´nucleotidase activities

The E-NTPDase activity in lymphocytes were determined as previously described by Leal et al. (2005), in which the reaction medium contained 2.0 mM of substrate (ATP or ADP), 0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 60 mM glucose, and 50 mM Tris-HCl buffer at pH 8.0 with final volume of 200 µL. Twenty microliters of cell resuspended in saline solution (2–4 µg of protein) were added to the reaction, and incubated for 70 min at 37 °C. Nucleotidase activities in heart homogenates were determined using the method described by Rosemberg et al. (2010), modified by Doleski et al. (2016). Twenty µL of heart homogenate previously centrifuged at 2500 rpm for 10 min were added to a specific enzymatic reaction. The NTPDase reaction medium contained 1.0 mM of substrate (ATP or ADP), 5.0 mM $CaCl_2$, and 50 mM Tris-HCl buffer (pH 8.0). The reaction medium for 5´nucleotidase contained 1.0 mM of substrate AMP, 5.0 mM $MgCl_2$, and 50 mM Tris-HCl buffer (pH 7.5). NTPDase and 5´nucleotidase reaction was incubated at 37 ºC for 30 min. All reactions were stopped with 200 µL of 10% trichloracetic acid (TCA). The amount of released inorganic phosphate (Pi) was

assayed by a method previously described by Chan et al. (1986), using malachite green as the colorimetric reagent and KH_2PO_4 as the standard control. Controls were carried out by adding the enzyme preparation after TCA addition to correct non-enzymatic nucleotide hydrolysis. All samples were tested in triplicate and the specific activity was reported as nmol of Pi released/min/mg of protein.

ADA activity

E-ADA activity in lymphocytes (Giusti and Galanti, 1984), and ADA activity in homogenized heart (Giusti, 1974) were measured. These methods are based on the direct measurement of the ammonia released when the enzyme acts in excess of adenosine. Briefly, lymphocytes, and heart homogenates reacted with 21 mM of the substrate (adenosine), pH 6.5, and incubation was carried out for 1 h at 37 °C. The reaction was stopped by adding 106 mM and 167.8 mM of sodium nitroprussiate and hypochlorite solution. Ammonium sulfate (75 µM) was used as ammonium standard. All the experiments were performed in triplicate and the values of ADA activity in lymphocytes and heart homogenates were expressed in U/mg of protein.

Histopathology

At necropsy, a part the heart was collected and fixed in 10% buffered formalin, routinely processed and embedded in paraffin wax. Tissue sections were stained with hematoxylin and eosin (HE) for histopathological examinations. Heart sections were examined under an optical microscope in a blind way, and lesions were scored as mild, moderate, and severe.

Statistical analysis

Variables were expressed as mean ± standard errors mean (SEM). The data obtained were analyzed statistically by the Student's t test for independent samples. The effect of the number of lymphocytes on purinergic enzymes (E-NTPDase, E-5'nucleotidase, and E-ADA) was analyzed by linear correlation. Differences were considered significant when probability (P) was <0.05.

RESULTS

Course of infection: parasitemia and hemogram

Trypomastigotes of *T. cruzi* in infected mice were observed 4 days PI, and the peak of parasitemia occurred at day 10 PI (Fig. 1). During the experimental period no apparent clinical signs were observed.

Infected animals had a reduction in the number of total erythrocytes, hematocrit and hemoglobin concentration compared to uninfected mice (P<0.01; Table 1). These animals also showed a reduction in the number of total leukocytes, probably due to decreased number of neutrophils (P<0.01). The number of lymphocytes and eosinophils did not differ between groups (P>0.05), but the number of monocytes increased significantly in *T. cruzi* infected mice (P<0.01; Table 1).

NTPDase, 5´nucleotidase, and ADA activities

The NTPDase activity (ATP and ADP substrates) in lymphocytes increased significantly (P<0.01 and P<0.05, respectively) in mice infected by *T. cruzi* compared to uninfected (Fig. 2a, 2b). Similarly, ADA activity in lymphocytes also increased (P<0.05) in infected mice (Fig. 2c). No significant correlation (P>0.05) was observed between the number of lymphocytes and purinergic enzymes (E-NTPDase, E-5'nucleotidase, and E-ADA) in lymphocytes.

In the heart, NTPDase activity (ATP and ADP substrates) did not differ between groups (P>0.05; Fig. 3a, 3b). The 5'nucleotidase activity (P<0.001) in the heart was reduced in infected animals compared to uninfected (Fig. 3c), unlike ADA activity that showed increased activities in the heart of infected animals (P<0.05; Fig 3d).

Histopathology

Macroscopically, no cardiac changes were observed. Histologically, the heart of mice experimentally infected by *T. cruzi* showed several pseudocysts containing amastigotes within cardiomyocytes, and necrosis associated with diffuse moderate to severe inflammatory infiltrate of lymphocytes (Fig. 4).

DISCUSSION

Anemia and leukopenia are findings already described in infections caused by *T. cruzi* (Cardoso and Brener 1980; Marcondes et al. 2000), as observed in this work. Associated with these alterations, enzymes of the purinergic system showed increased activities in lymphocytes of mice infected by *T. cruzi*, and they are responsible for regulating the levels of adenine nucleotides and nucleosides. Therefore, these changes trigger a mechanism that participates in the modulation of Chagas disease, primarily related to inflammation. The increase in ATP hydrolyze by E-NTPDase can be an anti-inflammatory effect of the enzyme, due to ATP, a pro-inflammatory molecule (Bours et al. 2006). The increase in E-NTPDase activity triggers a cascading effect, which was probably responsible for the increase in other enzymatic activities in lymphocytes. The increased E-ADA activity reduces the levels of adenosine, an anti-inflammatory molecule (Bours et al. 2006), and the E-ADA may have acted as an inflammatory marker.

ATP and ADP is a mediator, released into the bloodstream, in response to tissue injury and other pathological conditions, such as inflammation, hypoxia and ischemia (Bours et al. 2006). Increased ATP hydrolysis by E-NTPDase to ADP and AMP characterized an anti-inflammatory effect, such as pro-inflammatory molecule, that have increased their levels in consequence of *T. cruzi* infection, which caused inflammation of the heart, due to the presence of the parasite. Thus, increasing E-NTPDase activity reduces the extracellular levels of ATP, leading a reduction in late-releasing pro-inflammatory cytokines (Bours et al., 2006), which protects the tissue from the oxidative damage, and the down-production of oxygen radicals in animals infected by *T. cruzi.* According to the literature, E-NTPDase (ATP and ADP as substrates) activity was decreased in lymphocytes from patients with the indeterminate form of Chagas disease (IFCD) (Souza et al., 2012). The stage of infection can be directly related to the enzymatic alterations, as we evaluate the acute phase, and Souza et al. (2012) evaluated the chronic phase. However, increased E-NTPDase activity in lymphocytes may also have occurred in chagasic humans after infection. Acute infection by *T. evansi* reduced E-NTPDase activity in the first 10 days of infection, but showed a significant increase 15 days PI (Oliveira et al., 2012). Therefore, enzymatic changes of E-NTPDase activities might be related to the progression of the infection caused by trypanosomes, and thus, be involved in modulating the inflammatory response. According in literature, lymphocytic ecto-nucleotidase activity helps to understand this complex mechanism, since increased E-NTPDase activity controls high ATP interacting with P2 receptors in the cell membrane, and decreased E-ADA activity stimulates the interaction of low Ado concentration with its P1 receptors.

In cardiac tissue, NTPDase activity did not differ between groups, however, a large significant reduction in the 5'-nuceotidase activity was found to be of great interest. This reduction could reduce adenosine, one molecule with anti-inflammatory effect, that could maintain high levels of ATP and ADP in the heart, where there is an inflammatory process due to the presence of the parasite. Therefore, the authors believe that 5'-nucleotidase played a pro-inflammatory effect in acute *T. cruzi* infection, because A2a and A2b receptors couple to stimulatory G proteins and typically suppress cell responses by upregulating intracellular AMP levels (Junger 2011). Cells need these autocrine amplification mechanisms to tailor their functional responses to various extracellular cues. Depending on which purinergic receptor subtypes participate in specific purinergic signalling complexes, autocrine amplification mechanisms can act as checkpoints that enhanced or inhibit activation in response to different extracellular cues (Junger 2011). According to the literature, host cells release adenine nucleotides into the extracellular space, where these mediators are converted by NTPDase and 5'nuceotidase into adenosine; which is an anti-inflammatory in the short term but may also promote dermal, heart, liver, and lung fibrosis with repetitive signaling under defined circumstances (Ferrari et al., 2016). In 2012, researchers have drawn attention to a critical role for 5'-nucleotidase as a modulator of brain inflammation and immune function (Petrovic-Djergovic et al., 2012), which can also occur in the heart of chagasic mice in the acute phase of the disease as observed in this study. For these authors in their study verified that chimeric mice lacking 5'-nucleotidase in tissue had larger cerebral infarct volumes and more tissue leukosequestration than did mice lacking 5'-nucleotidase on circulating cells, and these data show a cardinal role this enzyme in suppressing ischemic tissue leucosequestration (Petrovic-Djergovic et al., 2012).

Increase in E-ADA activities in lymphocytes, and ADA in cardiac tissue might be a physiological response to reduce the concentrations of extracellular adenosine, an important nucleoside for different cellular functions. An interaction between adenosine and its ubiquitous receptors has anti-inflammatory effects, such as inhibition of the Th1 immune response (Cordero et al., 2001). Therefore, the increase in ADA activity suggests a decrease in adenosine levels in the extracellular medium, increasing the inflammatory response in an attempt to combat proliferation of the agent, and therefore, avoiding excessive tissue damage (Burnstock, 2006; Desrosiers et al., 2007). E-ADA activity was decreased in lymphocytes from IFCD patients (Souza et al., 2012), similarly to what occurred in serum of rats chronically infected by *T. cruzi,* using a clinical strain (Da Silva et al., 2011). In our study, there was no change in the number of lymphocytes in infected mice Y strain of *T. cruzi*, but the E-ADA activity was

increased in these cells. This was an expected result, since an adaptive immune responses are slow to develop on first exposure to a new pathogen, as specific clones of B and T cells have to become activated and expand; it can therefore take a week or so before the responses are effective (Alberts el at., 2002). However, in the *T. evansi* infection, researchers observed a negative correlation between lymphocyte number and activity of E-ADA in these cells on the fourth day of infection; this had become a lymphocytosis and decreased E-ADA activity (Da Silva et al 2011b). According to the authors, in this case the enzyme had an anti-inflammatory effect in the acute phase, in order to reduce injuries. However, with the progression of the disease (day 20 PI), there was an increased enzymatic activity (Da Silva et al 2011b), similarly to that observed in this study for the infection with *T. cruzi* on day 12 PI. The ADA inflammatory effect on cardiac tissue aimed to activate an inflammatory process that is mediated by ATP (pro-inflammatory molecule), among others, to eliminate the parasite. However, this event may be those responsible for the cardiac lesions observed in the acute phase of the disease.

In conclusion, the activities of E-NTPDase and E-ADA in lymphocytes, and cardiac 5'-nucleotidase and ADA in mice experimentally infected by *T. cruzi* were altered. Activation of NTPDase can reduce ATP levels, an important inflammatory mediator, so this enzyme shows an anti-inflammatory effect. The ADA also had increased activity; however, by hydrolyzing adenosine, an anti-inflammatory molecule, this enzyme may have inflammatory effect. Generally, these alterations may implicate in the pathophysiology of Chagas disease related of inflammatory response.

Ethical Committee

The present study was approved by the Ethics Committee for Use of Animals (CEUA) of the Universidade Federal de Santa Maria (UFSM) under protocol number 8288290615.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgments

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Table 1. Mean and standard error of hematological variables (erythrocytes, hemoglobin and hematocrit) and leukocytes (total leukocytes, lymphocytes, neutrophils, monocytes, eosinophils, and rods) in mice experimentally infected by *Trypanosoma cruzi*.

	Uninfected (control)	Infected	P
RBC ($x10^6/\mu L$)	7.5 ± 0.4	5.4 ± 0.3	<0.01
HGB (g/dL)	13.7 ± 0.5	10.7 ± 0.5	<0.01
HCT (%)	35.7 ± 2.2	25.8 ± 1.5	<0.01
WBC ($x10^3/\mu L$)	6.06 ± 0.6	2.18 ± 0.6	<0.01
Lymphocytes (%)	73.2 ± 1.6	73.0 ± 1.5	>0.05
Neutrophils (%)	21.8 ± 1.1	13.0 ± 0.9	<0.001
Rods (%)	0.4 ± 0.2	0.2 ± 0.1	>0.05
Eosinophils (%)	0.4 ± 0.2	0.4 ± 0.2	>0.05
Monocytes (%)	4.2 ± 0.6	13.4 ± 0.8	<0.001

Variables analyzed statistically by the Student’s t test for independent samples and expressed as mean ± standard errors mean (SEM).

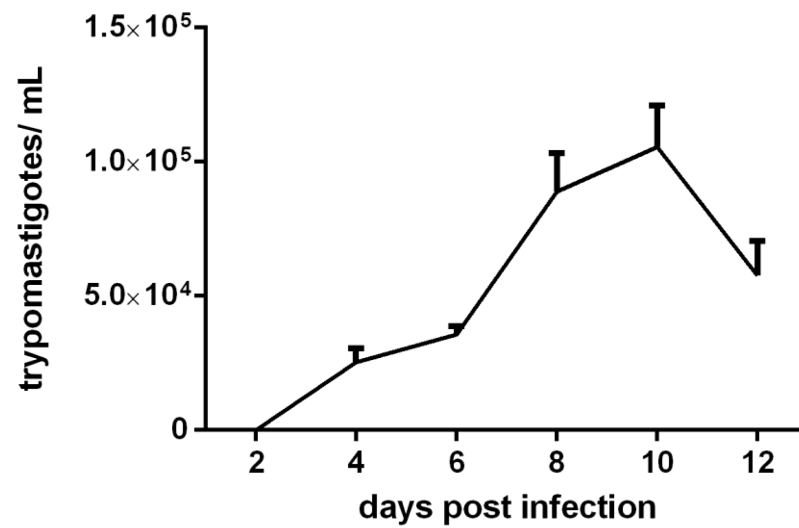

Figure 1. Parasitemia of mice infected by *Trypanosoma cruzi* (Y strain) during 12 days of the experiment.

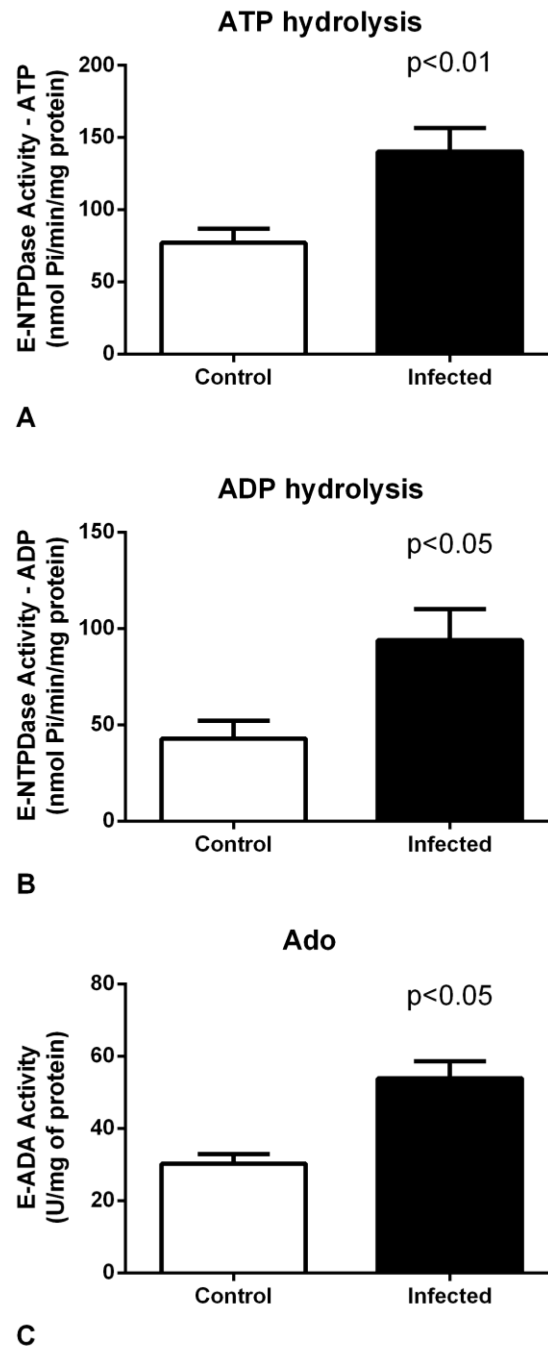

Figure 2. ATP (A) and ADP (B) hydrolysis by E-NTPDase and adenosine (C) desamination by E-ADA in lymphocytes of mice experimentally infected and uninfected (control) by *Trypanosoma cruzi*. The results were expressed as mean and standard error (SEM). Student's t test for independent samples was used for all the analyses.

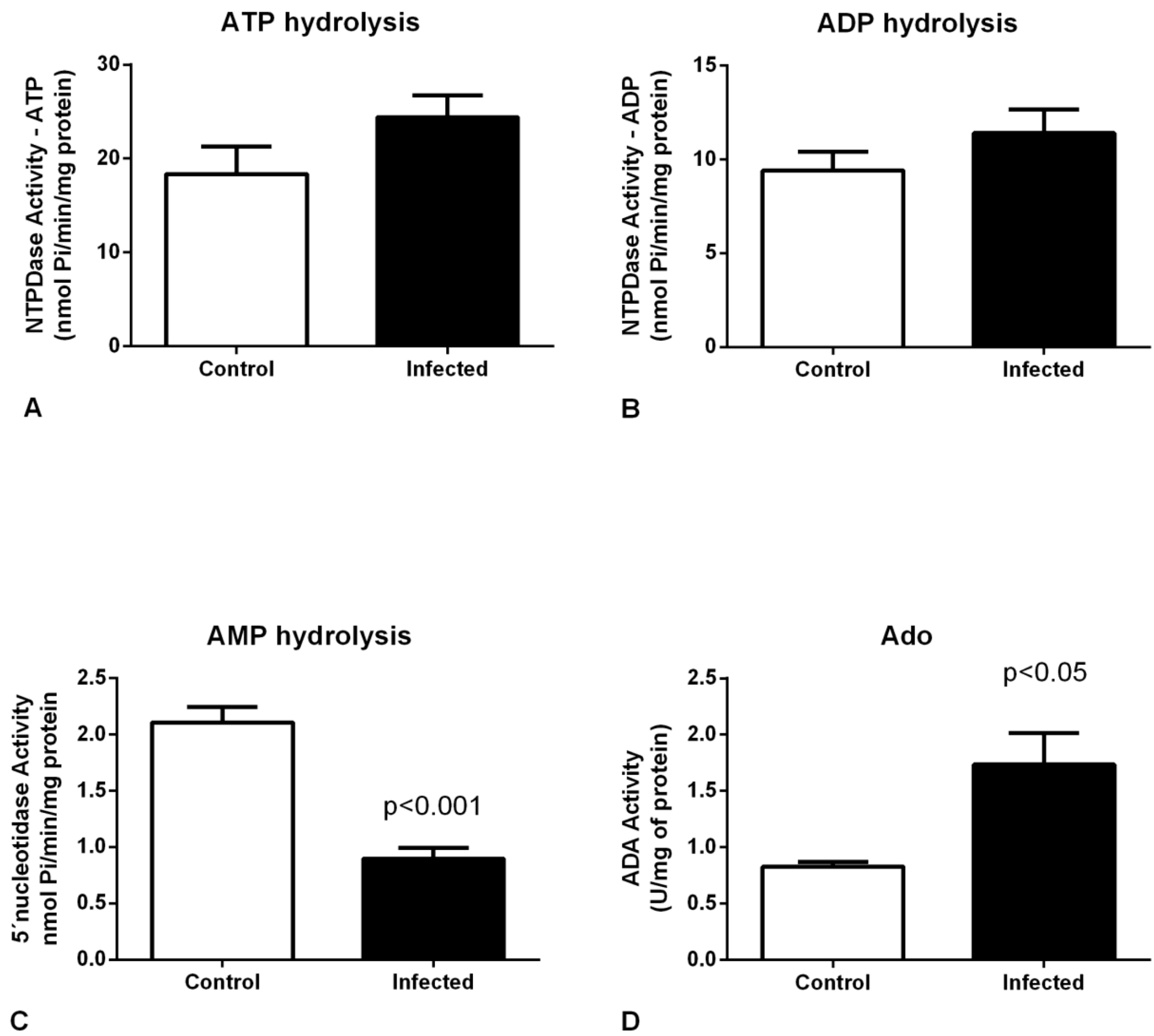

Figure 3. ATP (A), and ADP (B) hydrolysis by NTPDase, AMP (C) hydrolysis by 5'nucleotidase, and adenosine (D) desamination by ADA in heart of mice experimentally infected and uninfected (the control group) by *Trypanosoma cruzi*. Results were expressed as mean standard error of the mean (SEM). Student's t test for independent samples was used for all the analyses.

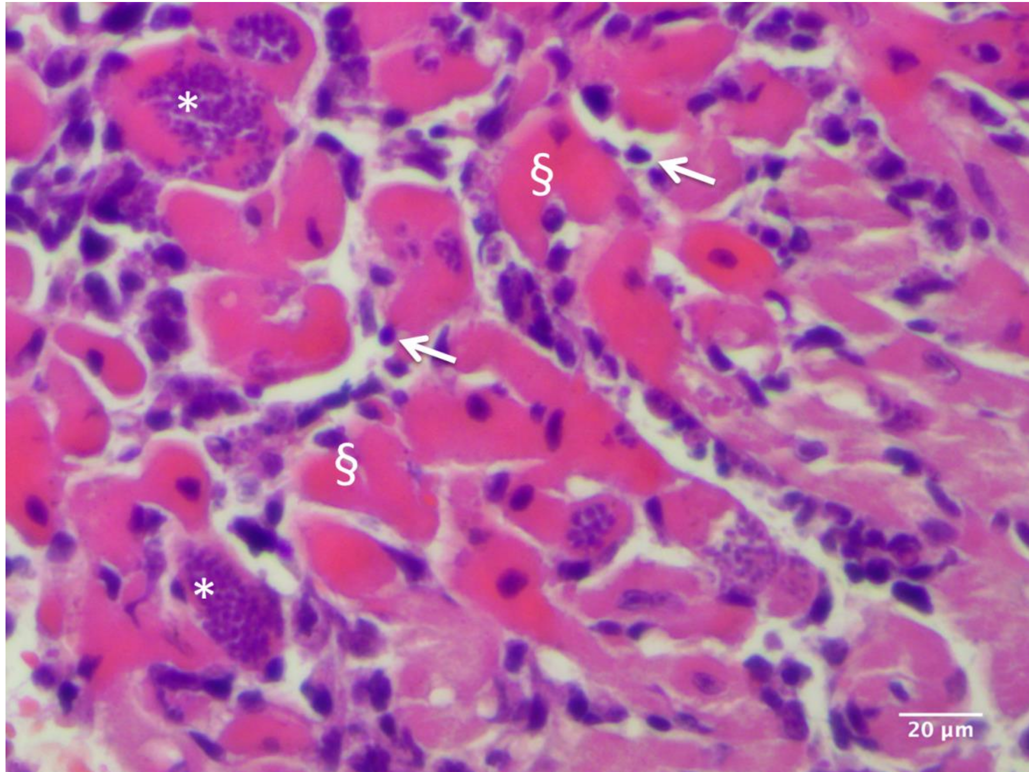

Figure 4. Cardiac histopathology of mice experimentally infected by *Trypanosoma cruzi*. Multiple pseudocysts containing amastigotes within cardiomyocytes (*). Focally extensive severe necrosis (§) associated with diffuse moderate to severe inflammatory infiltrate of lymphocytes (arrows). HE, Obj. 40 X.

4.2 ARTIGO II

Publicado na revista: **Molecular and Cellular Biochemistry**

Purinergic ecto-enzymes participate in the thromboregulation in acute in mice infection by *Trypanosoma cruzi*

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Abstract

Coagulation disorders have been described in Chagas disease with thrombocytopenia as an important event. Several mechanisms may be related to this pathogenesis, such as enzymes of the purinergic system, purine and receptors involved in the regulation and modulation of physiological events related to hemostasis. Therefore, the aim of this study was to evaluate the activities of E-NTPDase, E-5'nucleotidase, and ecto-adenosine deaminase (E-ADA) in platelets of mice experimentally infected by *Trypanosoma cruzi*. Twelve female mice were used, divided into two groups (n=6): uninfected and infected. Mice of infected group were intraperitoneally inoculated with 10^4 trypomastigotes of *T. cruzi* (strain Y). On day 12 post-infection (PI), blood samples were collected for quantitation and separation of platelets. A significant reduction in the number of platelets of infected mice ($P<0.05$) was observed. The activities of E-NTPDase (ATP and ADP substrates), E-5'nucleotidase, and E-ADA in platelets increased significantly ($P<0.05$) in mice infected by *T. cruzi* compared with uninfected animals. A negative correlation ($P<0.01$) was observed between the number of platelets and ATP hydrolysis (r= -0.64), and ADP hydrolysis (r= -0.69) by E-NTPDase. Therefore, there is a response from the purinergic system activating ectoenzymes in platelets of mice *T. cruzi* infected, as a compensatory effect of thrombocytopenia.

Keywords: Chagas disease; coagulation disorders; purinergic ectoenzymes.

Introduction

The involvement of the purinergic system in coagulopathies is known [1,2]. The purinergic signaling system plays an important regulatory role in cellular activation, blood flow, and vascular thrombosis by extracellular biomolecules, such as adenine nucleotides and their derivative nucleoside adenosines [3]. According to the literature, the adenine nucleotides ATP, ADP and AMP, and the nucleoside adenosine regulate and modulate many cellular functions, including platelet aggregation [1]. It is important to highlight that the ADP is primarily responsible for promoting this aggregation, while adenosine is a potent inhibitor [4,5]. Its anti-aggregant effect is mediated via G-protein coupled adenosine receptors (P1 purinoceptores), specifically the A2B adenosine receptor subtypes [6]. Therefore, enzymatic activities of E-NTPDase, E-5'-nucleotidase, and adenosine deaminase (E-ADA) are associated with essential mechanisms for thromboregulation and homeostasis [7], by regulating ATP, ADP, AMP, and adenosine [3]. Reports indicate that purinergic enzymes are associated to thrombocytopenia in trypanosomosis [7], that can also occur in Chagas disease, due to disorders in coagulation events [8].

Chagas disease is caused by *Trypanosoma cruzi*, a parasitic illness that infects the blood and several tissues of the host [9]. Humans highly infected show the indeterminate form of the disease, which is characterized by long periods without symptoms of the disease. Severe cardiac and/or digestive lesions in the chronic phase of the disease are common [10,11], such as cardiac arrhythmias, congestive heart failure, and thromboembolic events, as well as chronic cardiomyopathy [12, 13, 14]. Therefore, considering that purinergic enzymes are involved in the modulation of hemostasis of events related to the coagulation cascade, the aim of this study was to evaluate the activities of E-NTPDase, E-5'nucleotidase, and E-ADA in platelets of mice experimentally infected by *T. cruzi* related to thromboregulation.

Materials and methods

Strain

This study used blood trypomastigotes of *T. cruzi* (strain Y) [15] maintained in the Veterinary Parasitology Laboratory of the Universidade Federal de Santa Maria (UFSM) cryopreserved in liquid nitrogen.

Animal model and experimental design

Twelve female mice (65 days of age, 25 g of body weight) were used in this experiment. Mice were maintained at a constant temperature (23 ± 1 ºC) on a 12 h light/dark cycle with free access to feed and water and divided into two groups (n=6 each): uninfected and infected. The infected group was inoculated by intraperitoneal route with 10^4 blood trypomastigotes of *T. cruzi* from a mice previously infected. The uninfected group received saline by route.

Blood parasitemia

Parasitemia level was determined by counting the number of motile parasites in five microliters of fresh blood collected from the lateral tail vein as recommended by Brener [16], and the number of parasites was recorded every 2 days from 4 to 12 days post-infection and expressed as parasites/mL of blood.

Sample collection and platelet isolation

The animals were anesthetized with isofluorane and humanely euthanized by cardiac puncture using a syringe and needle 12 days PI. The blood collected was stored in tubes containing citrate as anticoagulant for platelet isolation and platelet quantification using an automatic counter Coulter T890® (Coulter Electronics, Inc, Hialeach, FL, USA).

Platelet isolation was prepared from approximately 3 mL of blood collected in tubes with 0.129M sodium citrate, according to the method described by Pilla et al. [17], and modified by Lunkes et al. [18]. Peripheral blood was centrifuged at 1000 rpm for 10 min resulting in a platelet-rich plasma (PRP) that was harvested and centrifuged at 3700 rpm for 30 min. The centrifugation of PRP resulting in a pellet of platelets which was suspended in 3.4mM of HEPES buffer (pH 7.0). Protein in platelet was measured by the Comassie Blue method, using serum albumin as standard. Heart fragment was separated for histopathological examination.

E-NTPDase, and E-5´-nucleotidase activities

The E-NTPDase enzymatic assay was carried out in a reaction medium containing 5 mM $CaCl_2$, 100 mM NaCl, 4 mM KCl, 5 mM glucose, and 50 mM Tris–HCl buffer, pH 7.4, at a final volume of 200 µL, as described by Lunkes et al. [18]. For AMP hydrolysis, the medium used was as previously described by Lunkes et al. [18], except that the 5 mM CaCl2 was replaced by 10 mM $MgCl_2$. Twenty microliters of the enzyme preparation (8–12 µg of protein) was added to the reaction mixture and incubated for 10 min at 37 °C. The reaction was initiated by the addition of ATP or ADP at a final concentration of 1.0 mM, and AMP at a final

concentration of 2 mM. The time of incubation was 60 min. Both enzymatic assays were stopped by the addition of 200 µL of 10% tricloroacetic acid (TCA) to provide a final concentration of 5%. Subsequently, the tubes were chilled on ice for 10 min. Released inorganic phosphate (Pi) was assayed by the method of Chan et al. [19] using malachite green as the colorimetric reagent and KH_2PO_4 as a standard. Controls were carried out to correct for non-enzymatic hydrolyses of nucleotides by adding enzyme preparation after TCA addition. All samples were tested in triplicate. Specific enzymatic activities were reported as nmol Pi released/min/mg of protein.

E-ADA activity

ADA activity in platelets was measured by the method of Giusti and Galanti [20], based on the direct measurement of ammonia formed when ADA acts in excess of adenosine. Briefly, platelets reacted with 21 mM of the substrate (adenosine), pH 6.5, and incubation was carried out for 1 h at 37 °C. The reaction was stopped by adding 106 mM and 167.8 mM sodium nitroprussiate and hypochlorite solution. Ammonium sulfate (75 µM) was used as ammonium standard. All the experiments were performed in triplicate and the values of ADA activity in platelets were expressed as U/mg of protein.

Histopathological analyses

Heart fragments were collected and fixed in 10% buffered formalin and embedded in paraffin wax. Tissue sections were stained with hematoxylin and eosin (HE) for histopathological examinations.

Statistical analysis

Variables were expressed as mean ± standard errors mean (SEM). The data obtained were analyzed statistically by the Student's t test for independent samples. The effect of the number of platelets on the purinergic enzymes was analyzed by linear correlation. Differences were considered significant when $P<0.05$.

Results

Course of infection: parasitemia and platelets number

The prepatent period in mice infected by *T. cruzi* occurred at day 4 post-infection (PI), and the peak of parasitemia occurred on day 10 PI (Fig. 1). During the experimental period, no

apparent clinical signs were observed. In addition, infected animals had a reduction in the number of total platelets compared to uninfected mice (P<0.05; Fig. 2).

E-NTPDase, E-5´-nucleotidase and E-ADA activities

The activity of E-NTPDase (ATP and ADP substrates) in platelets increased significantly (P<0.05 and P<0.01, respectively) in mice infected by *T. cruzi* compared to uninfected (Fig. 3a, b). E-5'-nucleotidase (P<0.01) and E-ADA activities (P<0.05) also increased in platelets of infected animals compared to uninfected (Fig. 3c, d).

A negative correlation was observed between the number of platelets and ATP hydrolysis (r= -0.64; P<0.01) and ADP hydrolysis (r= -0.69; P<0.01) by E-NTPDase. A negative correlation was also observed between the number of platelets and AMP hydrolysis (r= -0.73; P<0.01) by E-5'-nucleotidase and adenosine deamination (r= -0.81; P<0.01) by E-ADA (Fig. 4).

Histopathological analyses

The heart of mice experimentally infected by *T. cruzi* showed several pseudocysts containing amastigotes within cardiomyocytes, and necrosis associated with diffuse moderate to severe inflammatory infiltrate of lymphocytes (Fig. 5). No lesions were observed in uninfected animals.

Discussion

The pathological findings described in this study, such as thrombocytopenia as consequence of *T. cruzi* infection, have already been described by Cardoso and Brener [21] and Marcondes et al. [8]. Associated with these alterations, enzymes of the purinergic system showed increased activities in platelets of mice infected by *T. cruzi*, which are responsible for regulating the levels of adenine nucleotides and nucleosides. Therefore, these alterations trigger a mechanism that participates in the modulation of Chagas disease, primarily related to hemostasis. The increased in NTPDase activity triggered a cascading effect, which was probably responsible for the increase in other enzymes in platelets. The increased ADA activity reduces the levels of adenosine, an anti-platelet molecule [5].

In the literature, focal myocytolytic lesions and infiltration by mononuclear cells associated with platelet aggregates in the microcirculation determinate chagasic cardiomyophathy [9]. An increase in ATP hydrolysis (a pro-inflammatory molecule) observed in this study may have the purpose of reducing the concentration of this nucleotide adenine in

the extracellular medium, probably in order to minimize the inflammatory process, which would be harmful to the host. It notes that the role of ATP in coagulation disorders it is not clear [22]. However, an excessive platelet aggregation can occur in damaged vasculature as a consequence of inflammation, since mononuclear cells release potent platelet-aggregating factors (ADP; thromboxane A2 and platelet-activiting factor, PAF), contributing to the state of microvascular hypoperfusion, as evidenced in areas of myocardial damage in chagasic mice [23]. Therefore, we believe that the change in ATP hydrolysis is related directly to the inflammatory response, and not to disturbances in coagulation, which indirectly can influence the hydrolysis of other nucleotides, like in the purinergic cascade, as seen in chagasic mice. It is worth mentioning that in a study of chronic chagasic patients, the activity of platelet NTPDase was not altered in the hydrolysis of ATP and ADP [9], unlike what occurred in this study in the acute infection by the *T. cruzi*.

An increase in NTPDase activity can trigger failure in platelet aggregation and vasoconstriction due to ADP decrease in the extracellular face of platelets [24], and these results corroborate to the reduction of platelets (Fig. 2) in animals infected in this study. The thrombocytopenia was also observed in other studies with *T. cruzi* [8,21,25], and therefore, the Chagas disease alters hemostasis mechanisms, which may hinder the implementation of some physiological functions of the body. In rats infected by *Trypanosoma evansi*, there is a reduction in ADP hydrolysis by NTPDase in platelets in the first ten days of infection, but a significant increase on day 15th PI [7]. Unfortunately, since this variable was not evaluated in this study at the beginning of infection, we do not know if the enzymatic behavior was the same for different species of the parasites. However, according to the literature, this change shows the involvement of enzymes in the thromboregulation. These mechanisms, as well as thrombocytopenia are related to the activation of the enzyme as a compensatory mechanism to the chronicity of the disease [7, 22].

The increase in 5'-nucleotidase activity in platelets may have decreased the extracellular concentrations of AMP, and the increase in E-ADA activity in platelets might be a physiological response to decreased concentrations of extracellular adenosine, an important nucleoside for different cellular functions. The 5'-nucleotidase and E-ADA have demonstrated the involvement of these enzymes in the thromboregulation mechanisms, and altered enzymatic activities have been reported in many diseases [7,26,27], and a similar increase was also verified in E-5'-nucleotidase activity in platelets of the indeterminate form of Chagas disease (IFCD) in humans [9]. In trypanosomosis by *T. evansi* in rats [7], as well as in dogs experimentally infected by *Rangelia vitalii* [22] reduced activity in E-ADA in platelets was observed, another

disease that has well marked coagulation disorders, such as thrombocytopenia and bleeding. Our study found increased E-ADA activity in the acute infection, which might be a physiological response to reduced concentrations of extracellular adenosine, an important nucleoside for different cellular functions. According to the literature, adenosine is an important modulator of vascular tone, and it is a well-known inhibitor of platelet aggregation [28,29], therefore, by analyzing this effect separately, we can attribute the increase in E-ADA as a compensatory effect in order to avoid hemorrhages or severe thrombocytopenia.

According to researchers, E-NTPDase is the major enzyme regulating nucleotide metabolism at the surface of vascular smooth muscle cells and thus contributes to the local regulation of vascular tone by nucleotides [30]. Endothelial cells, and activated platelets release adenine nucleotides (ATP and ADP) in a non-lytic manner [30], and this release occurs in response to mechanical constraints, in pathological situations that occur in *T. cruzi* infections. Therefore, this increase in ATP and ADP release by platelets from infected mice by *T. cruzi* may have increased E-NTPDase activity. As it is a purinergic cascade, the increase in ATP hydrolysis will increase the hydrolysis of ADP and AMP, as well as adenosine deamination by E-ADA in acute phase of disease. In conclusion, we observed an increase in E-NTPDase, E-5'-nucleotidase and E-ADA activities in platelets of mice experimentally infected by *T. cruzi*. Furthermore, there was a negative correlation between platelet count and the activity of purinergic enzymes, i.e., thrombocytopenia caused an increase in the activity of these enzymes as a compensatory effect on the regulation of hemostasis. Therefore, we conclude that the purinergic enzymes are involved in thromboregulation in acute infection by *T. cruzi* in an experimental model, as previously observed in IFCD patients [9].

Ethical Committee

The present study was approved by the Ethics Committee for Use of Animals (CEUA) of the Universidade Federal de Santa Maria (UFSM) under protocol number 8288290615.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgment

Professor Mario Steindel from the Protozoology Laboratory of Universidade Federal de Santa Catarina (UFSC) for providing the *T. cruzi* strain used in this study. The funding agencies

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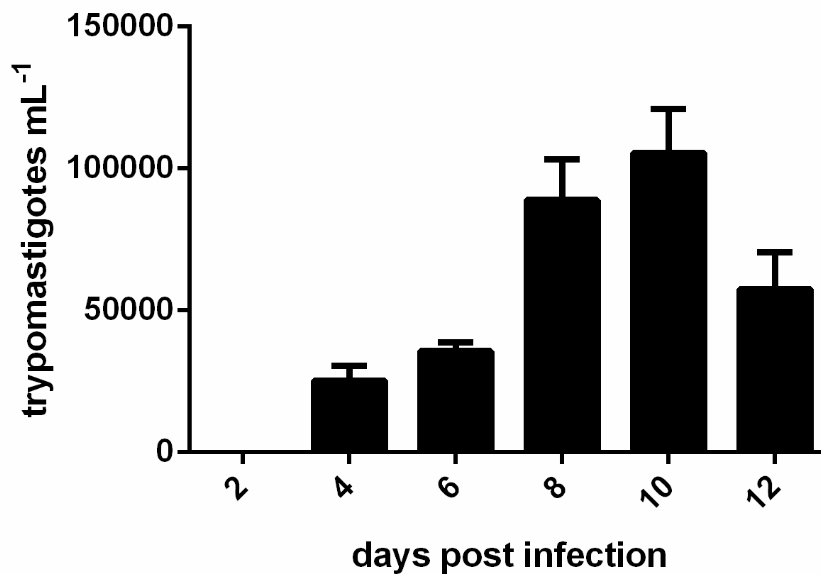

Fig. 1 Parasitemia of mice infected by *Trypanosoma cruzi* (Y strain) during 12 days of the experiment.

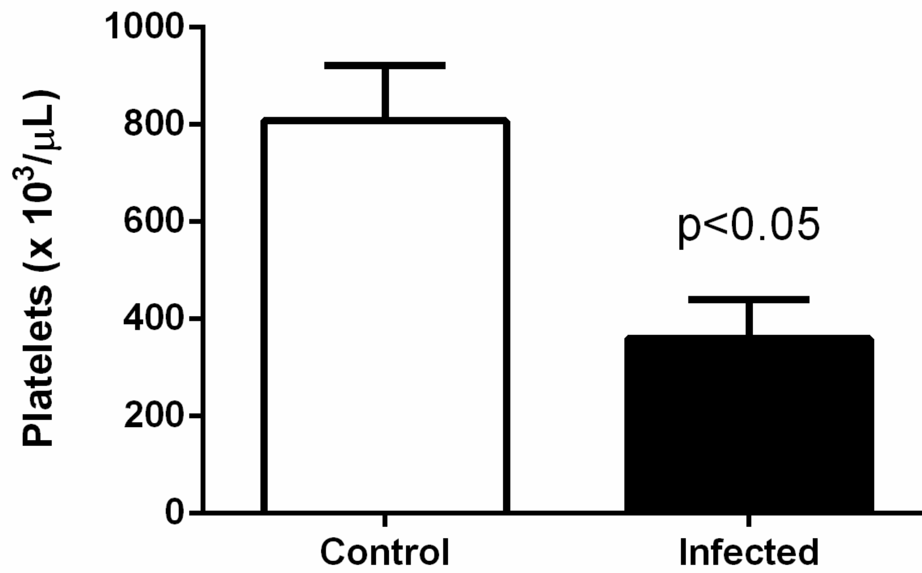

Fig. 2 Platelet counts in animals experimentally infected by *Trypanosoma cruzi* after 12days. Variable analyzed statistically by the Student's t test for independent samples and expressed as mean ± standard errors mean (SEM).

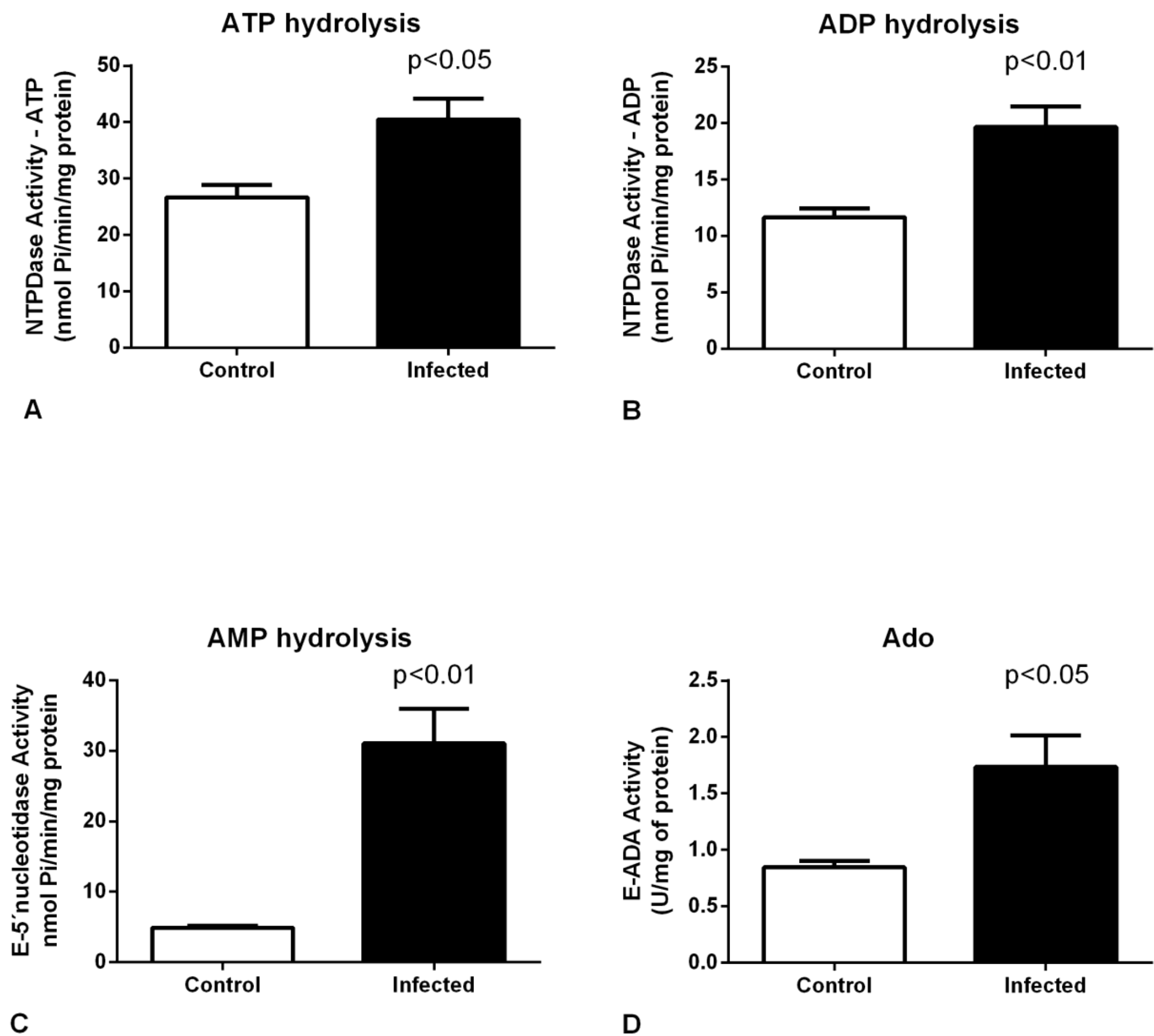

Fig. 3 ATP (A), and ADP (B) hydrolysis by E-NTPDase, AMP (C) hydrolysis by 5'nucleotidase, and adenosine (D) desamination by E-ADA in platelets of mice experimentally infected by *Trypanosoma cruzi.* Results were expressed as mean standard error of the mean (SEM). Student's t test for independent samples was used for all analyses.

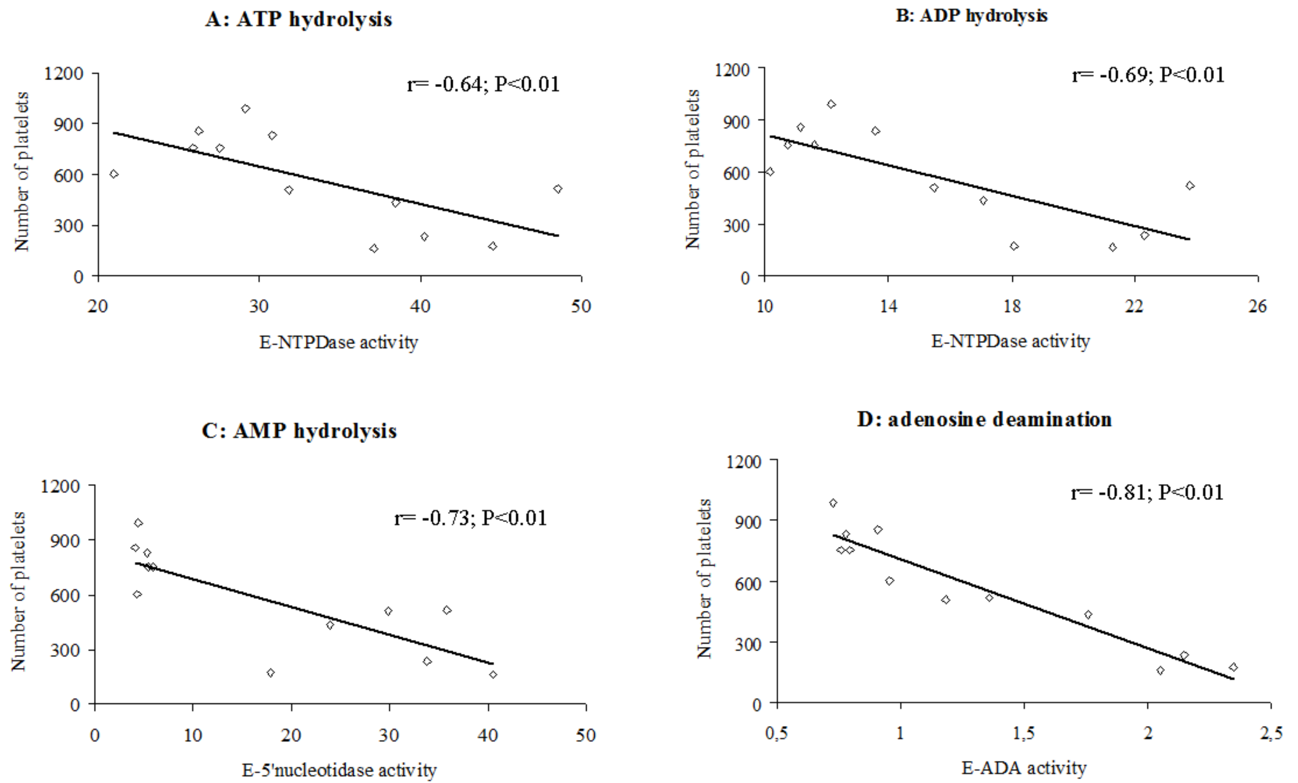

Fig. 4 Correlation between number of platelets (x10^3) on ATP (A), and ADP (B) hydrolysis by E-NTPDase, AMP (C) hydrolysis by 5'nucleotidase, and adenosine (D) desamination by E-ADA in platelets of mice experimentally infected by *Trypanosoma cruzi.*

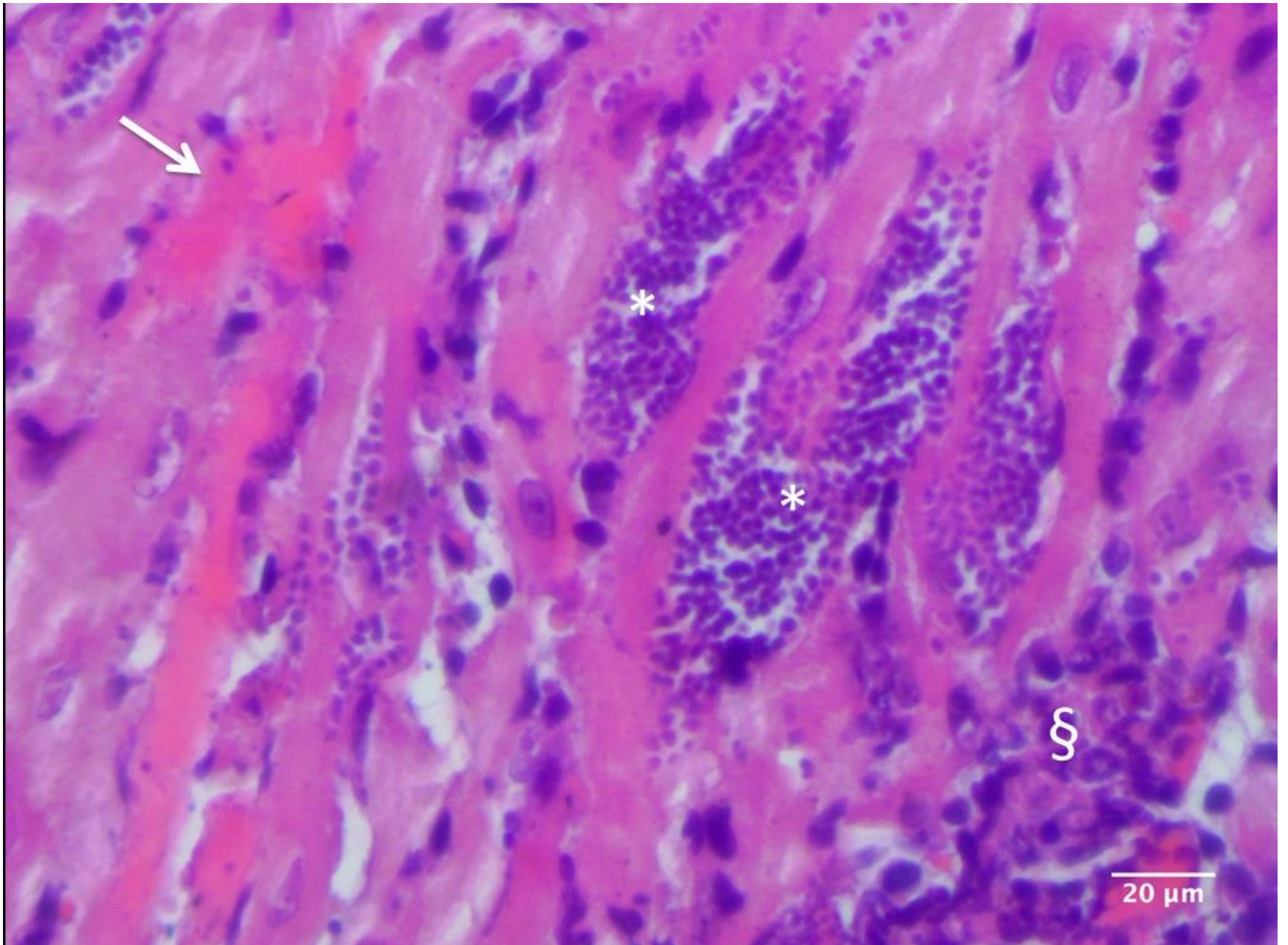

Fig. 5 Histopathology of heart of rats experimentally infected by *Trypanosoma cruzi*. Multiple pseudocysts containing amastigotes (*), multifocal mild to moderate necrosis (arrow) associated with multifocal mild to moderate inflammatory infiltrate of lymphocytes (§). HE, Obj 40 X.

4.3 ARTIGO III

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Treatment with 3′-deoxyadenosine and deoxycoformycin in mice infected by *Trypanosoma cruzi* and its side effect on purinergic enzymes

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Abstract

The aim of this study was to evaluate the efficacy of 3'-deoxyadenosine and deoxycoformycin combination in the treatment of mice infected by *T. cruzi*, as well as to verify the influence of the treatment on purinergic enzymes. Heart and serum samples were collected from 60 mice (30 infected and 30 uninfected) at day 12 post-infection. To verify treatment efficacy, parasitemia was monitored, and the treatment with 3'-deoxy adenosine and deoxycoformycin combination was able to reduce it, but had no curative effect on mice. Seric activities of NTPDase (ATP and ADP substrate) and ADA were increased significantly in untreated mice infected by *T. cruzi* compared to the negative control, as well as mice treated with 3'-deoxyadenosine and deoxycoformycin (alone or combined) modulated the activity of NTPDase (ATP and ADP substrate), preventing them from increasing in infected animals (activity similar to healthy animals). Treatment with deoxycoformycin alone and associated with 3'-deoxyadenosine modulated the activity of ADA preventing them from increasing in infected animals. However, seric activities of ADA in mice treated with 3'-deoxyadenosine (cordycepin) alone does not modify the ADA activity compared with infected and non-treated mice. However, the 5'-nucleotidase activity decreased significantly in infected untreated animals and the same occurred with infected and treated animals deoxycoformycin and with 3'-deoxyadenosine. However, treatment with deoxycoformycin associated with 3'-deoxyadenosine preventing them from decreasing the 5'-nucleotidase activity. Therefore, we conclude that the treatments did not have curative success for mice infected by *T. cruzi*. However, the treatments were able to modulate the purinergic enzymes during the infection by *T. cruzi*, which may contribute to reduce the inflammatory damage in heart.

Keywords: *Trypanosoma cruzi*; cordycepin**;** pentostatin; purinergic enzymes; pathogenesis.

1. Introduction

Chagas disease is a zoonosis caused by the protozoan *Trypanosoma cruzi* and was first described in 1909 [1]. This is a neglected disease in the world and remains a serious health problem with approximately 8–10 million people infected in the world [2]. The main drugs used for the treatment of Chagas disease are benznidazole and nifurtimox, despite their significant side effects [3]. In Brazil, benznidazole is available to treat patients with approximately 80% of effectiveness in the acute phase of the disease, and <20% of the patients with chronic Chagas disease [4,5].

An analogue product of purine, 3'-deoxyadenosine (cordycepin), was effective to cure mice infected by *Trypanosoma brucei* in both the acute and chronic phases (with central nervous system involvement) of the disease [6,7,8]. Another study tested the same treatment and the same dose in mice infected by *Trypanosoma evansi*, and obtained 100% curative efficacy [9,10]. Besides that, the biological effect of cordycepin against *T. brucei*, *T. cruzi,* and *Leishmania* sp. was also observed *in vitro* [6]. According to the literature, this effect is due to an inability of trypanosomes to engage in new purine synthesis, which has been exploited as a therapeutic target [11].

The cordycepin combined with pentostatin has been used to treat parasitic diseases. This adenosine analogue can perform similar effect to adenosine that is present in all tissues of mammals, demonstrating important functions related to cell signaling, neuroprotection, thromboregulation, and immune processes [12, 13]. In addition, adenosine has anti-inflammatory action, playing a central role in inflammation and immunomodulation [14]. The concentration of extracellular adenosine is regulated by the ADA activity, which is considered an enzyme in the purine metabolism, catalyzing the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively [15]. It is noteworthy that ADA is one of the purinergic enzymes, that together form a purinergic cascade, and therefore, when one enzyme is affected it can directly influence the behavior of the other. Among the other enzymes, we highlight the NTPDase and 5'nucleotidase, responsible for regulating the levels of extracellular ATP, ADP and AMP [16], and involved in several physiological functions of the organism, but with important regulatory participation (inflammatory, hemostasic among others) in pathological conditions [17]. Therefore, the aim of this study was to evaluate the efficacy of the 3'-deoxyadenosine and deoxycoformycin combination in the treatment of mice infected by *T. cruzi*, as well as to verify the influence of the treatment on purinergic enzymes.

2. Materials and Methods

2.1 Chemicals

The substrates ATP, ADP, adenosine, as well as Trizma base, Coomassie Brilliant Blue G and bovine serum albumin were obtained from Sigma Chemical Co (St. Louis, MO, USA) and K_2HPO_4, from Reagent. All the other chemicals used in this experiment were of high purity.

2.2 Drugs

3'-deoxyadenosine (Cordycepin®) was obtained from Sigma Chemical Co (St. Louis, MO, USA). Deoxycoformycin (Pentostatin®) obtained from Tocris Bioscience (Minneapolis, MN, USA). Unless otherwise indicated, all reagents were diluted in PBS, aliquoted and stored at –20 ºC until further use. Benznidazole (LAFEPE, Recife, Brazil) dissolved in DMSO (Sigma–Aldrich, St. Louis, MO, USA) was used as reference drug in the anti-*T. cruzi* assays.

2.3 *Trypanosoma cruzi* strain

This study used blood containing trypomastigotes of *T. cruzi* strain Y [18] maintained in the Veterinary Parasitology Laboratory of the Universidade Federal de Santa Maria (UFSM), Brazil.

2.4 Animal model

Sixty female (Swiss) mice (45 days, 20 – 30 g) from the Central Animal House of the Universidade Federal de Santa Maria (UFSM) were used in this experiment. The animals were maintained at a constant temperature (23 ± 1 ºC) on a 12 h light/dark cycle with free access to food and water.

2.5 Mice infection and treatment

Each mouse was inoculated by intraperitoneal route with 0.2 mL containing $1x10^4$ trypomastigotes of *T. cruzi* (strain Y) using blood from mice previously infected. After inoculation, the animals were randomly assigned to five groups of six animals each. Other five groups were formed by uninfected mice.

In order to verify treatment efficacy, as well as the effect of the drugs on purinergic enzymes, the groups were formed as follows: the Group A – consisted of uninfected mice; the Group B – consisted of uninfected and treated mice with cordycepin (2 mg/kg); the Group C –

consisted of uninfected and treated mice with pentostatin (0.2 mg/kg); the Group D – composed by uninfected and treated mice with a combination of cordycepin (2 mg/kg) and pentostatin (0.2 mg/kg); the Group E – consisted of uninfected and treated mice with benznidazole (100 mg/kg); the Group F – consisted of infected and untreated mice; the Group G - formed by infected and treated mice with cordycepin (2 mg/kg); the Group H - formed by infected and treated mice with pentostatin (0.2 mg/kg); the Group I - formed by infected and treated mice with a combination of cordycepin (2 mg/kg) and pentostatin (0.2 mg/kg); the Group J - formed by infected and treated mice with benznidazole (100 mg/kg). The treatments started five days after inoculation with the administration of the cordycepin and pentostatin (for 3 consecutive days) and benznidazole (for 3 consecutive days). The doses of cordycepin and pentostatin used in this study were based on studies with *T. brucei* and *T. evansi* [6,10].

2.6 Parasitemia estimation

The infection was monitored by counting the number of motile parasites in 5 µL of fresh blood sample drawn from the lateral tail vein, as recommended by standard protocol [19]. The number of blood trypomastigote forms of *T. cruzi* was recorded every 2 days from 4 to 12 days post-infection (PI) and the number of trypanosomes was expressed as parasites/mL.

2.7 Sample collection

Due to treatment ineffectiveness, based on the results of parasitemia (Figure 1), we defined day 12 PI as the end of the experiment. On day 12 PI, the animals were anesthetized with isoflurane in an anesthetic chamber, and bled by cardiac puncture. The blood collected was stored in tubes to obtain serum. Thereafter, animals were euthanized by decapitation following recommendations of the Ethics Committee.

2.8 Histopathology

At necropsy, the heart was collected and fixed in 10% buffered formalin and embedded in paraffin wax. Tissue sections were stained with hematoxylin and eosin (HE) for histopathological examination. The sections were evaluated by a specialist in Veterinary Pathology at the Federal Institute of Santa Catarina (IFC). Heart sections were viewed under an optical microscope in a blind way and lesions were described (lesions classified as mild, moderate and severe).

2.9 Seric NTPDase and 5´nucleotidase activities

NTPDase and 5´nucleotidase activities in serum samples were determined as previously described by [20]. The reaction mixture for the NTPDase activity contained 3mM of ATP or ADP as substrate and 112.5 mM Tris-HCl (pH 8.0). The reaction mixture for 5´nucleotidase was composed of 3 mM of AMP as substrate and 100 mM of Tris-HCl (pH 7.5). The reaction mixtures were incubated with approximately 1.0 mg of homogenized protein at 37ºC for 40 min on a final volume of 0.2 mL. The reaction was stopped by the addition of 0.2 mL of 10% trichloroacetic acid (TCA). All samples were centrifuged (5000 g for 5 min) to eliminate precipitated protein, and the supernatant was used for the colorimetric assay. The samples were chilled on ice and the amount of released inorganic phosphate (Pi) was measured by the method of [21]. In order to correct non-enzymatic hydrolysis, control samples were used by adding the homogenate after the reaction was stopped with TCA. Enzymatic activities were expressed as nanomoles of Pi released per min per milligram of protein (nmol of Pi/min/mg protein).

2.10 Seric ADA activity

ADA activity was measured spectrophotometrically in serum samples by the method of [22]. The reaction was started by the addition of the substrate (adenosine) to a final concentration of 21 mmol/L, and incubation carried out for 1 h at 37 °C. The reaction was stopped by adding 106 mmol/L/0.16 mmol/L phenol-nitroprusside/mL solution. The reaction mixtures were immediately mixed to 125 mmol/L/11 mmol/L alkaline hypochlorite (sodium hypochlorite) and vortexed. Ammonium sulfate 75 µmol/L was used as ammonium standard. The ammonia concentration is directly proportional to the absorption of indophenol at 620 nm. The specific activity was reported as U/mg of protein in serum.

2.11 Statistical analysis

The data met the assumption of a parametric testing according to the Kolmogorov-Smirnov test. Statistical analysis of parasitemia in the same day between groups was performed using one-way analysis of variance (ANOVA) followed by the Tukey post-hoc analysis. The effect on purinergic enzymes (NTPDase, 5'nucleotidase, and ADA) were analyzed by bilateral two-way analysis of variance (ANOVA) followed by the Tukey post hoc test for comparison of means. Differences between groups were rated significant at $p < 0.05$. Variables were expressed as mean ± standard errors mean (SEM).

3. Results

3.1 Course of infection

Trypomastigotes of *T. cruzi* in infected mice (the group F) were observed 4 days PI, and the peak of parasitemia occurred on day 10 PI (Fig. 1). On day 6 PI, a significant reduction on parasitemia ($p<0.001$) was observed in the group of infected and benznidazole treated animals (the group J) compared to other groups; and day 8 PI these groups did not present trypomastigotes in the blood. A significant decrease ($p<0.001$) on parasitemia was observed in groups treated with cordycepin (the group G), pentostatin (the group H), and cordycepin associated with pentostatin (the group I) on days 8 and 10 PI compared to infected and untreated mice. However, 12 days PI only the group G reduced significantly ($p<0.001$) compared to the infected control.

3.2 Seric NTPDase, 5´-nucleotidase and ADA activities

NTPDase activity (ATP and ADP substrates) and E-5'-nucleotidase in serum did not differ between groups in uninfected mice (groups A, B, C, D and E) ($p>0.05$; Fig. 2a, b, c). However, seric ADA activities in serum of uninfected mice reduced significantly in the groups C and D ($P<0.01$) compared to the group A (Fig 2d).

The activity of seric NTPDase (ATP and ADP substrate) increased significantly ($p<0.001$, and $P<0.05$, respectively) in mice infected by *T. cruzi* (the group F) compared to the group A (Fig. 3 a, b). However, the groups G, H, I and J did not differ from the negative control group (the group A) to NTPDase enzyme (ATP and ADP substrate) (Fig. 3a, b). Seric E-5'-nucleotidase activity decrease significantly ($p<0.01$) in infected animals (the group F), the group G ($p<0.05$) and the group H ($p<0.05$) compared to the group A (Fig. 3c). However, the others group of infected and treated animals did not differ from the negative control group (the group A) (Fig.3c). The ADA activity in serum increased significantly ($p<0.001$) in mice infected by *T. cruzi* (the group F) compared to the group A, as well as the group G. The seric ADA activity in the groups H, I and J did not differ from the group A (Fig. 3d).

3.3 Histopathology

Macroscopically, no cardiac changes were observed in 36 mice of this experiment. However, histological examination of heart samples of mice experimentally infected by *T. cruzi* (the group F) showed pseudocysts containing amastigotes within cardiomyocytes, mild to severe necrosis associated with mild to severe multifocal inflammatory infiltrate of

lymphocytes (Fig. 4). The changes between mild to moderate necrosis associated with mild to moderate multifocal inflammatory infiltrate of lymphocytes were observed in groups G, H, I. However, we did not observe histological cardiac changes in the group treated with benznidazole (the group J), which indicates that heart damage was prevented.

4. Discussion

In this study, a potent *in vivo* trypanocidal action of cordycepin against *T. cruzi* was observed, similarly as observed in some experiments using mice infected by *T. evansi* [9,10] and *T. brucei* [6]. Moreover, cordycepin and pentostatin used alone or combined, but mainly the associated treatment, were able to reduce the degree of cardiac inflammatory infiltrates.

In vivo, the treatment with cordycepin exerted a similar trypanocidal effect compared to benznidazole, demonstrating that this treatment may be considered an important approach to treat mice infected by *T. cruzi.* On the other hand, a study conducted by Dalla Rosa et al. [10] demonstrated that the association between cordycepin and pentostatin showed 100% efficacy against *T. evansi,* a fact not observed in this study.

The purinergic system develops an important role in the immune and inflammatory responses during many parasite infections, such as observed in humans infected by *T. cruzi* [23,24]. In the present study, a significantly increase of seric NTPDase and ADA activities was observed in mice infected by *T. cruzi,* while the seric 5'-nucleotidase activity decreased. The augmentation on NTPDase (ATP as substrate) may be explained as an attempt to decrease the ATP levels, an important pro-inflammatory molecule when present in excessive amounts [25], i.e., this response may contribute to restrict the inflammatory damage, similarly to what was observed during the infection caused by *Fasciola hepatica* [17]. The ADP nucleotide is mainly related to thrombocytopenia and platelet aggregation [16], important parameters related to pathogenesis of Chagas disease [26]. In this sense, an increase of seric NTPDase activity (ADP as substrate) may occur as an attempt to reduce ADP levels, an important molecule linked to platelet aggregation, and consequently, this enzyme prevents or reduces hypercoagulation. Regarding the inhibition of seric 5'-nucleotidase activity, this may explain the increase on AMP levels during *T. cruzi* infection in humans [23], Finally, the increase on seric ADA activity may lead to a reduction on adenosine levels, an important anti-inflammatory molecule [27], that may contribute to inflammatory damage, in agreement with [23] while studying lymphocytes of humans infected by *T. cruzi.* In this study, we verified that untreated and pentostatin treated animals showed reduced ADA activities, since this drug is known as an inhibitor of this enzyme, a finding already described by [28].

Recently, a study conducted by [29] demonstrated that treatment with extracts of a plant called *Uncaria tomentosa* was able to improve the purinergic system, contributing to enhance the host immune system. Our study also demonstrated that the treatment with cordycepin and pentostatin affected seric nucleotidases and nucleosidase activities. We observed, for the first time, that cordycepin and pentostatin were able to prevent alterations on purinergic enzymes, which may have contributed to improve or module the immune response during *T. cruzi* infection, and consequently, prevent or reduce the inflammatory process. Recently, study conducted by Martinez et al. [30] demonstrated that purinergic signalling participates in the pathogenesis of cardiac alterations linked with induction of release of pro-inflammatory mediators, such as interleukin-17 and interferon gamma. In this sense, the use of cordycepin and pentostatin (alone or associated) can be an alternative to prevents or minimize the pro-inflammatory effects in heart tissue through the purinergic system modulation, since presented interesting results in the modulation of purinergic enzymes in serum. In addition, in uninfected animals, these two drugs, at the doses used, were not able to alter ADA and 5'nucleotidase activities, i.e. the treatment did not cause side effects in the purinergic enzymes.

The treatment with cordycepin and pentostatin (alone or associated) was unsuccessful to cure mice experimentally infected by the parasite. Therefore, differently from what was observed for *T. evansi* and *T. brucei*, this therapeutic protocol, at the dose used, should not be recommended to treat Chagas disease. On the other hand, the treatment was able to modulate purinergic enzymes in mice infected by *T. cruzi*, which may contribute to minimize the cardiac inflammatory infiltrates.

Ethical Committee

The present study was approved by the Ethics Committee for Use of Animals (CEUA) of the Universidade Federal de Santa Maria (UFSM) under protocol number 8288290615.

Conflict of interest

The authors have declared no conflict of interest.

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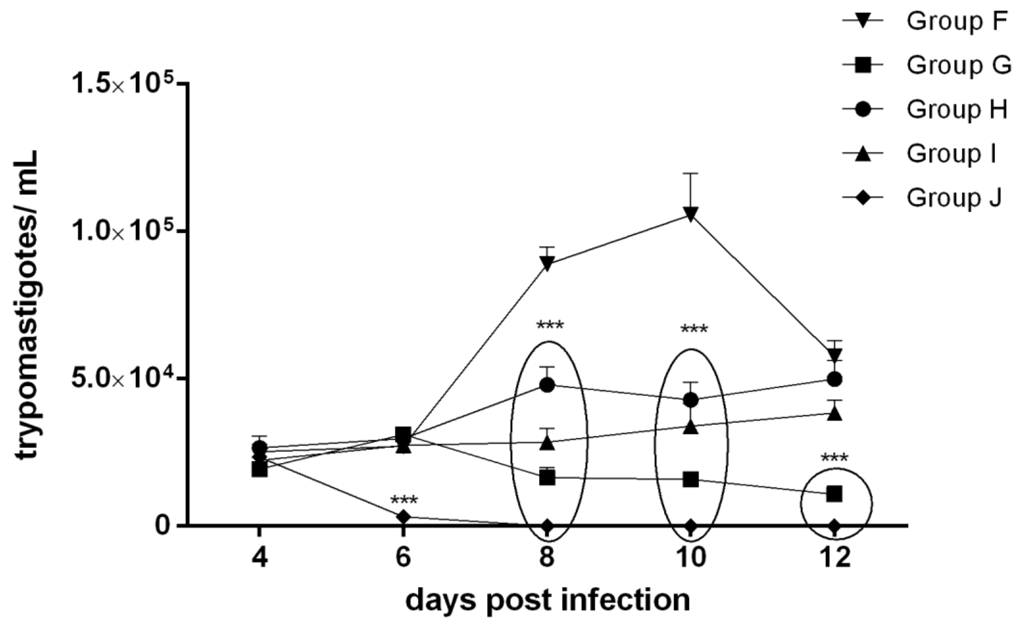

Fig. 1. *In vivo* trypanocidal effect evaluated by one-way analysis of variance (ANOVA) followed by the Tukey post-hoc analysis. *** $p<0.001$ compared to the control group.

Note: Infected control (the group F); Infected and treated with cordycepin (the group G); Infected and treated with pentostatin (the group H); Infected and treated with cordycepin and pentostatin (the group I); Infected and treated with benznidazole (the group J).

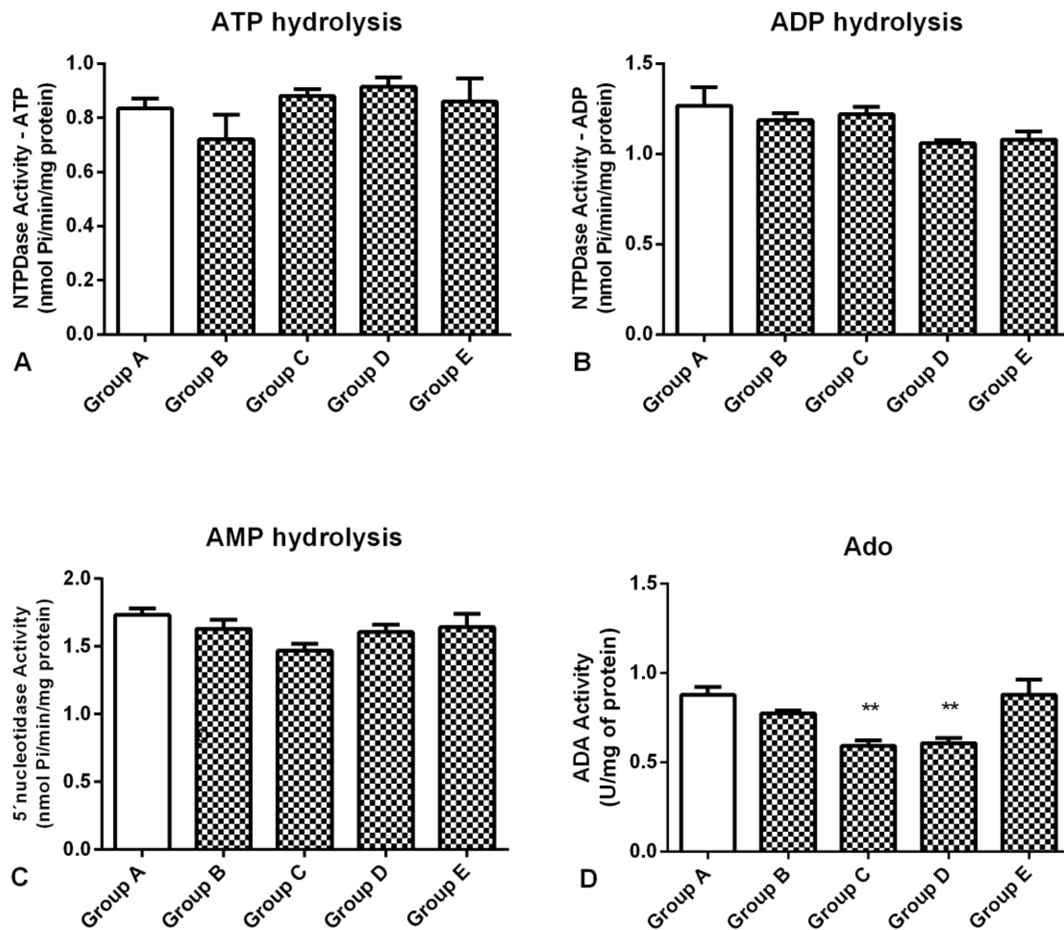

Fig. 2. Seric NTPDase (ATP [A] or ADP [B] as substrate), 5'-nucleotidase [C] and adenosine deaminase (ADA) [D] activities in uninfected mice evaluated by bilateral two-way analysis of variance (ANOVA) followed by the Tukey post hoc test. Results were expressed as mean standard error of the mean (SEM). ** $p<0.01$ compared to the group A. Note: Uninfected control (the group A); Uninfected and treated with cordycepin (the group B); Uninfected and treated with pentostatin (the group C); Uninfected and treated with cordycepin and pentostatin (the group D); Uninfected and treated with benznidazole (the group E).

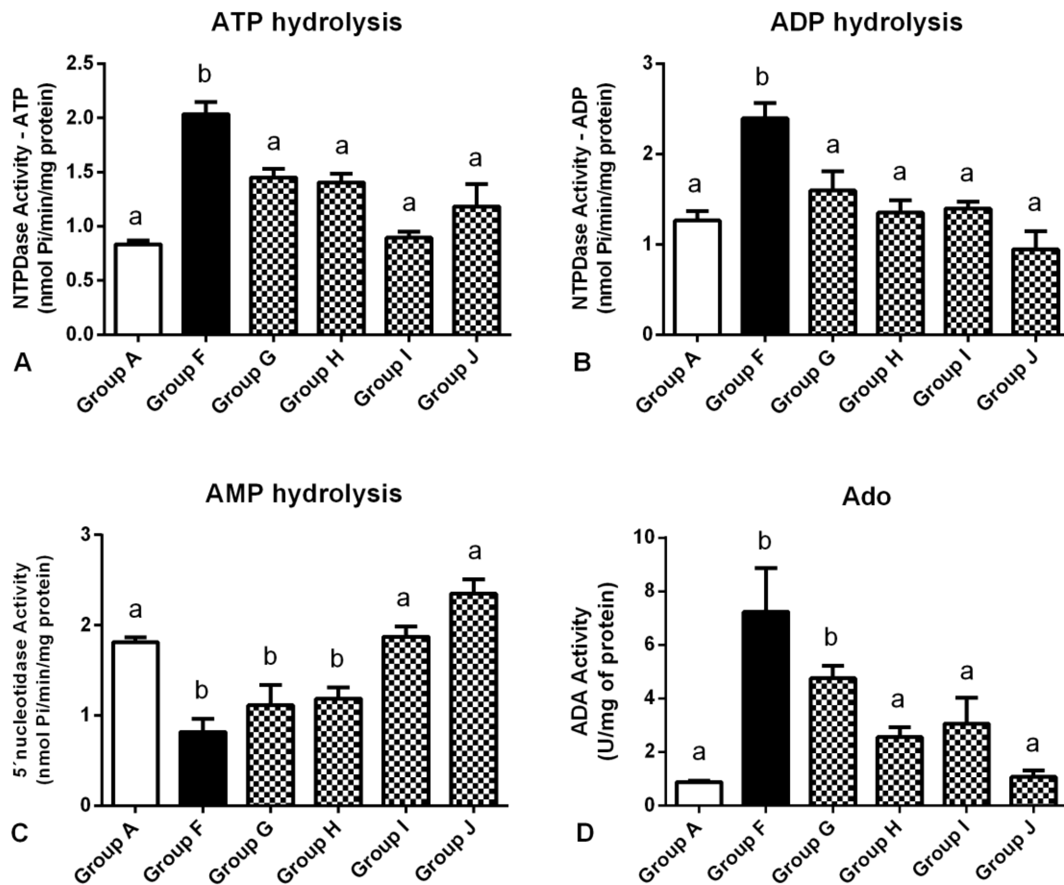

Fig. 3. Seric NTPDase (ATP [A] or ADP [B] as substrate), 5'-nucleotidase [C] and adenosine deaminase (ADA) [D] activities in mice experimentally infected by *Trypanosoma cruzi* (groups F to G) and uninfected mice (the group A) evaluated by bilateral two-way analysis of variance (ANOVA) followed by the Tukey post hoc test. Results were expressed as mean standard error of the mean (SEM). Same letters did not represent a significant difference. Note: Uninfected control (the group A); Infected control (the group F); Infected and treated with cordycepin (the group G); Infected and treated with pentostatin (the group H); Infected and treated with cordycepin and pentostatin (the group I); Infected and treated with benznidazole (the group J).

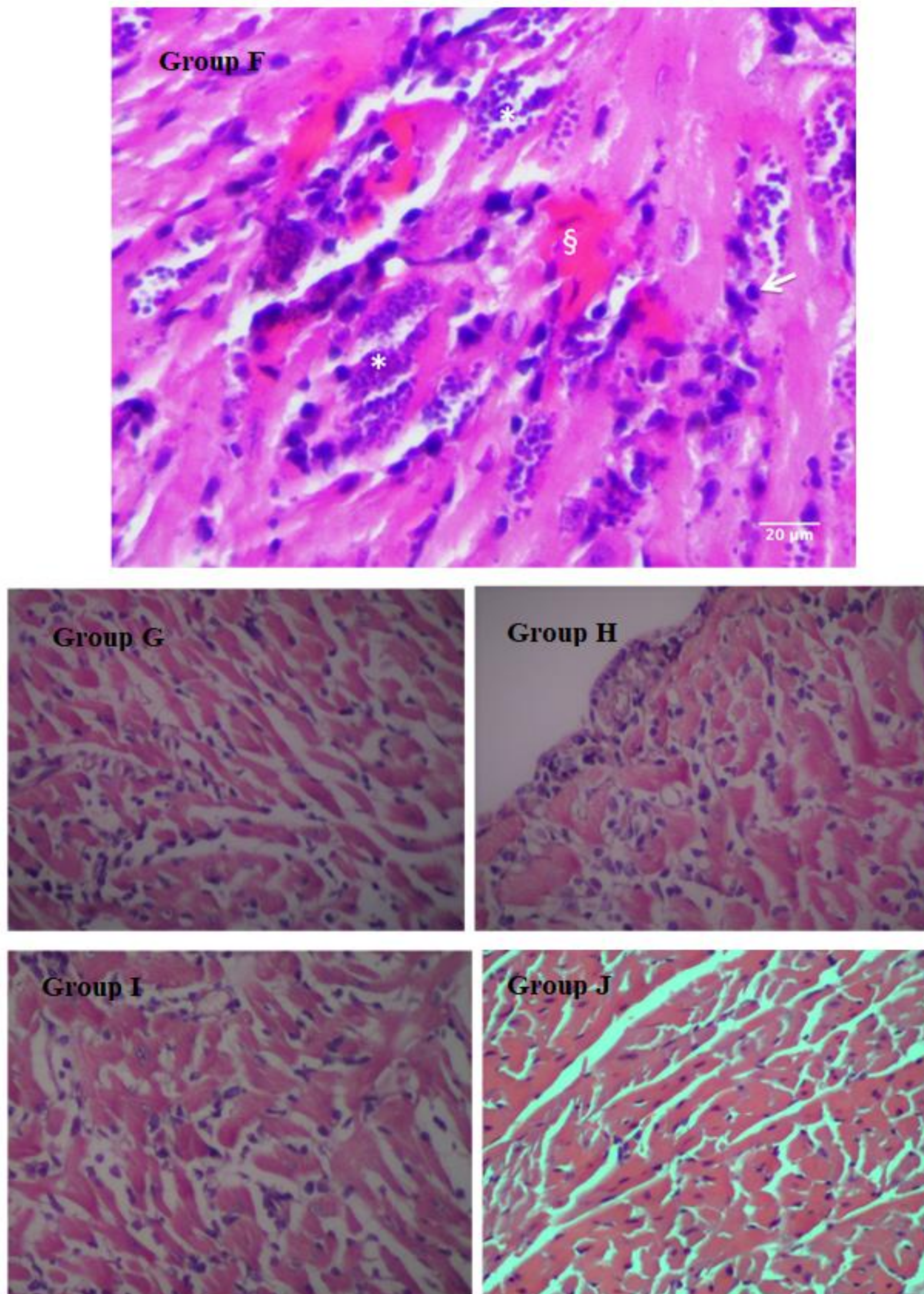

Fig. 4. Cardiac histopathology of mice experimentally infected by *Trypanosoma cruzi* (day 12 post-infection). Pseudocysts containing amastigotes (*), moderate multifocal necrosis (§) associated with mild to moderate multifocal inflammatory infiltrate of lymphocytes (arrow) in Group F; Inflammatory infiltrate in the heart of animals of groups G, H and I; Cardiac tissue without lesion in group J (HE, Obj 40 x). Note: Infected control (the group F); Infected and treated with cordycepin (the group G); Infected and treated with pentostatin (the group H); Infected and treated with cordycepin and pentostatin (the group I); Infected and treated with benznidazole (the group J).

4.4 ARTIGO IV

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Nucleotide and nucleoside involvement in immunomodulation in experimental Chagas' disease

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Abstract

The aim of this study was to evaluate whether *Trypanosma cruzi* infections cause alterations in the levels of seric purines, which could contribute to host immunomodulation. Twelve mice were divided into two groups identified as control (uninfected) and infected (*T. cruzi*) groups. The influence of the disease on seric purine levels was verified on day 20 post-infection (PI) by HPLC. Infected mice had circulating trypomastigotes during the experiment, as well as amastigote forms in the heart associated with inflammatory infiltrates. An increase on adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine (ADO), inosine (INO), and uric acid (URIC) levels was observed in infected animals, while the adenosine monophosphate (AMP) and xanthine (XAN) levels were reduced compared to mice of the control group, indicating a possible impairment on the purinergic system, and consequently, on the immune system during the clinical course of the disease. In summary, the *T. cruzi* infection alters the seric purine levels, and consequently, modulate the immune system.

Keywords: Chagas disease, nucleosides, nucleotides, purines, inflammatory response.

INTRODUCTION

Trypanosoma cruzi is the etiologic agent of Chagas disease, which is generally transmitted to humans through the fecal droppings of infected triatomine insects belonging to the Reduviidae family, being considered an important public health problem in Latin America, since the infection is endemic in this region [1]. Recently, the World Health Organization estimated that approximately seven million people are currently infected by *T. cruzi* in the endemic regions, with a risk to develop the chronic form of the disease [2]. Although the transmission by insects is considered the most important source of infection, other mechanisms of transmission are known, such as blood transfusions, congenital, and ingestion of contaminated sugarcane juice and açaí palm fruit, and organs for transplants [1,3].

In humans, heart diseases are the main consequence of Chagas disease, however other tissues and systems can be affected. Despite the wide variety of studies, the mechanisms are not yet fully understood as to how the parasite invades mammalian host cells. Recently, it was verified that acute mice infections caused by *T. cruzi* led to the involvement of enzymes of the purinergic system on the pathogenesis of the disease, participating in thromboregulation and inflammatory responses [4,5]. According to the literature, nucleosides and nucleotides of the purinergic system are responsible for a variety of biological functions in short-term and/or long-term signaling, including inflammation [6]. This signaling is mediated by ecto-nucleotidase family, that are responsible for controlling the levels of these nucleotides and nucleosides by sequential hydrolysis of adenosine triphosphate (ATP) into adenosine monophosphate (AMP), sequentially deamination of AMP into adenosine (ADO), adenosine into inosine (INO). Continuing, inosine is hydrolyzed into hypoxanthine (HYPO), that are hydrolyzed into xanthine (XAN). Lastly, XAN is hydrolyzed into uric acid, completing the purinergic cascade [6,7].

It is important to emphasize that theses nucleosides and nucleotides are involved in the pathophysiology of several parasitic infections [8], including others species of *Trypanosoma* [9]. These nucleosides are linked to the immune system response and inflammation, since ATP exerts a pro-inflammatory profile when in excessive amounts, since it acts on the purinoreceptors type P2. However, nucleoside ADO exerts an anti-inflammatory profile acting in the purinoreceptors P1, inducing the liberation of anti-inflammatory cytokines [7]. Therefore, the aim of this study was to evaluate whether *T. cruzi* infection causes alteration on seric levels of purine, which could contribute to host immunomodulation.

MATERIALS AND METHODS

Trypanosoma cruzi strain

This study used blood trypomastigotes of *T. cruzi,* Colombian strain [10], maintained cryopreserved in liquid nitrogen in the Veterinary Parasitology Laboratory of the Universidade Federal de Santa Maria (UFSM), Brazil.

Animal model and experimental design

Twelve female (Swiss) mice (45 days, 20 – 30 g) from the Central Animal House of the Universidade Federal de Santa Maria (UFSM) were used in this experiment. The animals were maintained at a constant temperature (23 ± 1 ºC) on a 12 h light/dark cycle with free access to food and water.

Six mice were inoculated by the intraperitoneal route with 0.2 mL of blood containing $4x10^3$ trypomastigotes of *T. cruzi* (Colombian strain) obtained from a mouse previously infected (the infected group); and other six animals were used as the control group (uninfected mice), which received saline to suffer the same experimental procedure of infected group.

The number of blood trypomastigotes of *T. cruzi* was recorded every 2 days from day 6 up to day 20 post-inoculation (PI), and the number of trypanosomes was expressed as parasites per mL. The amount of motile parasites in fresh blood samples drawn from the lateral tail vein was monitored as recommended by a standard protocol [11].

Sampling

On day 20 PI, the animals were anesthetized with isoflurane in an anesthetic chamber, and blood was collected by cardiac puncture. The blood was placed into tubes without anticoagulant and centrifuged (3.000 rpm for 15 min) to obtain serum samples that were stored at -80 ºC for purine analyses. Also, the heart was collected for histopathological analyses, as described below.

Seric purine levels

Purine compounds and metabolic residues were analyzed by HPLC according to Voelter et al. [12]. The proteins were denaturated by 0.6 mol/L of perchloric acid. All samples were centrifuged (16,000 x *g* for 10 min at 4ºC), and the supernatants were neutralized by the addition of 4.0 N KOH followed by clarification with a second centrifugation (16,000 x *g* for 30 min at

4ºC). Then, the supernatants were collected and centrifuged again (16,000 x *g* for 30 min at 4ºC). Aliquots of 20 µL were applied to a reversed-phase HPLC (LC-20AT model, Shimadzu, Kyoto, Japan) using a C_{18} column (Ultra C18, 25 cm x 4.6 mm x 5 µm, Restek – USA). The elution was carried out applying a linear gradient of 100% solvent A (60 mM of KH_2PO_4 and 5 mM of tetrabutylammonium chloride, pH 6.0) to 100% of solvent B (solvent A plus 30% of methanol) over a 40 min period (flow rate of 1.0 mL/min) according to a method previously described [12]. Mobile phases were filtered through a 0.45 µm Millipore filter prior to analysis, and all the reagent utilized were of HPLC grade. The amount of purine compounds and metabolic residues were measured by absorption at 254 nm. The retention time of the standards was used as a parameter for identification and quantification by comparison of the peak area. Purine levels (adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine (ADO), inosine (INO), hypoxanthine (HYPO), xanthine (XAN) and uric acid (URIC)) were expressed as nmol per mL.

Histopathology

Heart fragments were collected and fixed in 10% buffered formalin and embedded in paraffin wax. Tissue sections were stained with hematoxylin and eosin (HE) for histopathological examinations, and evaluated using a light microscope, as described in details by Do Carmo et al. [5].

Statistical analysis

The data were submitted to the normality test, which showed normal distribution. Then, statistical analysis was performed by analysis of variance (ANOVA) followed by the Student test. P values <0.05 were considered statistically significant.

RESULTS

Course of infection

During the experiments, the animals showed no apparent clinical signs of the disease. However, trypomastigote forms of *T. cruzi* were observed in the bloodstream 6 days PI. The peak of parasitemia occurred between day 10 PI (Figure 1).

Seric purine levels

Seric levels of purines were shown in Figure 2. Seric ATP and ADP levels increased significantly in infected animals compared to the control group ($P<0.05$). Unlike, the AMP levels were lower in infected group compared to control group ($P<0.05$). Seric adenosine and inosine levels increased significantly in infected animals compared to the control group ($P<0.05$). Xanthine levels were reduced in infected animals compared to uninfected animals ($P<0.05$). There was no difference in hypoxanthine levels between infected and control groups ($P>0.05$). The uric acid levels increased significantly in infected animals compared to the other group ($P<0.05$).

Histopathology

Macroscopically no cardiac changes were observed in all mice of the experiment. However, microscopically it was observed pseudocysts containing amastigotes, associated with mild to moderate multifocal inflammatory infiltrate of macrophages in the heart of mice experimentally infected by *T. cruzi* (the infected group) (Fig. 3). The animals of the control group did not show histological alterations in the heart.

DISCUSSION

In this study, we found increased seric concentrations of ATP, ADP and ADO, while the AMP levels decreased in infected animals, similarly to what was observed in animals experimentally infected by *T. evansi* [9]. These results might be linked to alterations in the enzymes of the purinergic system, such as NTPDase, 5'-nucleotidase, and ADA. A study conducted by Oliveira et al. [13] observed several alterations on these enzymes in platelets of animals infected by *T. evansi*, along with alterations in ATP, ADP and ADO levels. The increase in ATP levels may be related to an intense inflammatory response, since ATP is a pro-inflammatory molecule that acts on purinoreceptors P2, inducing the release of pro-inflammatory cytokines, that contribute to tissue damage [7]. The increase of seric ADP levels may be linked to the reduction of NTPDase activity, and thus, to the impairment of coagulation [14], since ADP is the final mediator of platelet recruitment and thrombus formation [15]. Thus, increased ADP levels could be associated to some coagulation disorders during infection, such as reported by Pinazo et al. [16].

A significant increase on ADO levels was observed in infected animals, a fact related to the anti-inflammatory profile, probably, as an attempt to reduce the damage, since ADO is a molecule with anti-inflammatory properties [17]. This finding could be related to the anti-inflammatory and anti-oxidative properties of cordycepin (an analogous adenosine) as described by Park et al. [18]. Moreover, a recent study conducted by Yuan et al. [19] demonstrated that cordycepin attenuates the inflammatory process through the reduction of pro-inflammatory mediators, such as nitric oxide, myeloperoxidase and interleukin-1, as well as it promotes an increase on anti-inflammatory molecules, such as interleukin-10. Thus, the analogous adenosine may prevent or reduce the inflammatory process.

Studies have shown that inosine, hypoxanthine, xanthine and uric acid have potent immunomudolatory, anti-inflammatory, and protective action [20, 21]. Thus, a significant increase on seric inosine and uric acid levels may be considered an attempt of the organism to minimize the intense inflammatory damage, which corroborates to our histopathological findings in cardiac tissues. Seric alterations could be related to increased xanthine oxidase and xanthine oxidorecuctase, responsible for the catabolism of hypoxanthine and xanthine to uric acid [21], similarly as observed during infections by *Haemonchus contortus* [22].

Therefore, *T. cruzi* alters the seric purine levels, and consequently, impairs the host immune system since increased ATP levels have direct consequences on the pro-inflammatory response, as well as increased adenosine has on the anti-inflammatory response, leading to an immunomodulation. We can also conclude that *T. cruzi* caused an increase on uric acid levels, a well-known potent antioxidant.

Compliance with Ethical Standards

The present study was approved by the Ethics Committee for Use of Animals (CEUA) of the Universidade Federal de Santa Maria (UFSM) under protocol number 8288290615, following the guidelines stablished by Brazilian Society of Science in Laboratory Animals.

Conflict of interest

None

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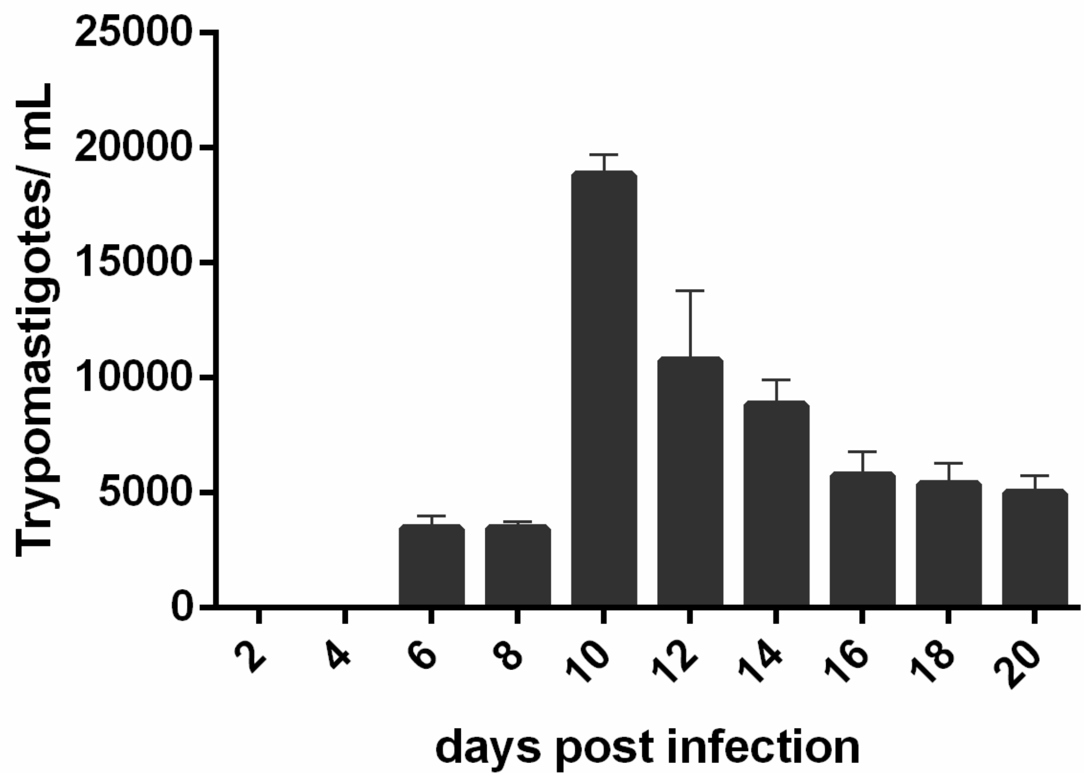


Figure 1: Parasitemia in mice experimentally infected by *Trypanosoma cruzi* (the infected group).

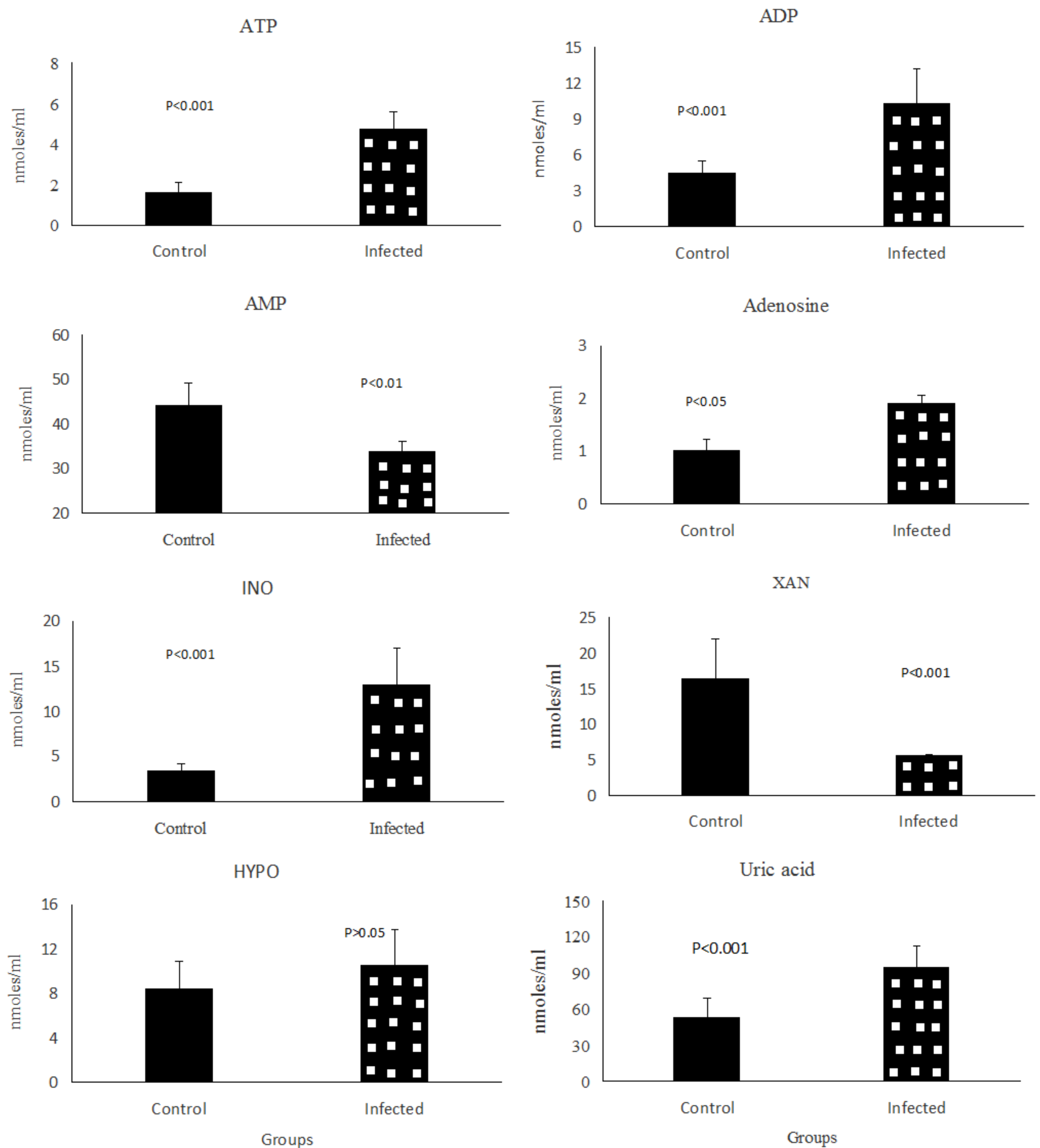
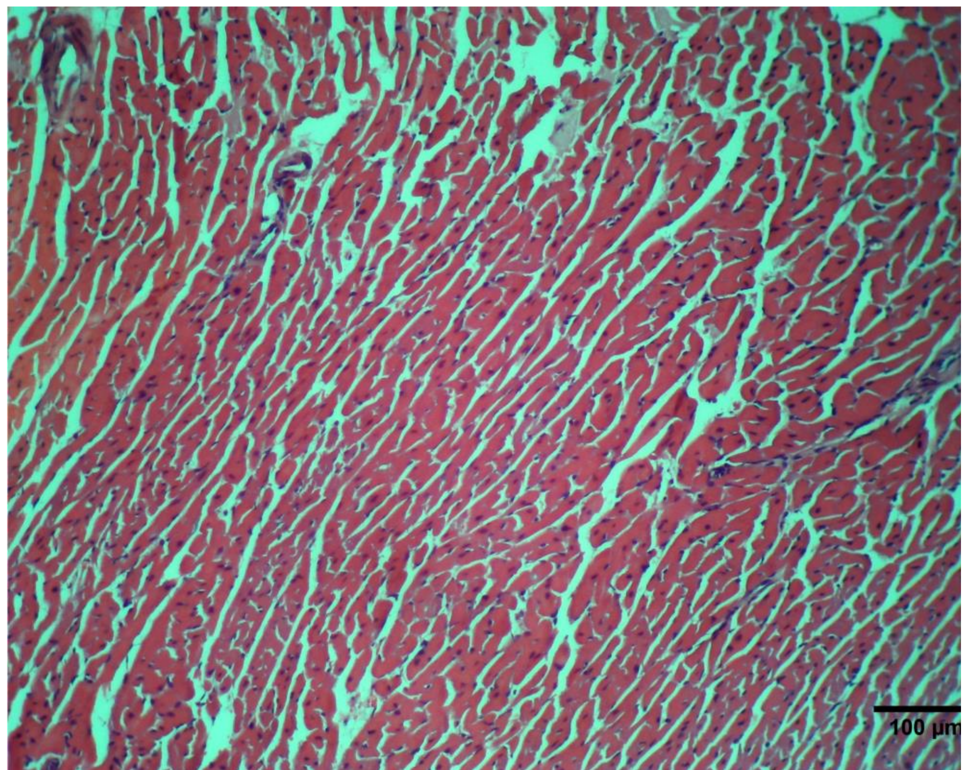


Figure 2: Seric purine levels of mice experimentally infected by *T. cruzi*: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine (ADO), inosine (INO), hypoxanthine (HYPO), xanthine (XAN) and uric acid (URIC). Groups statistically different ($P < 0.05$).



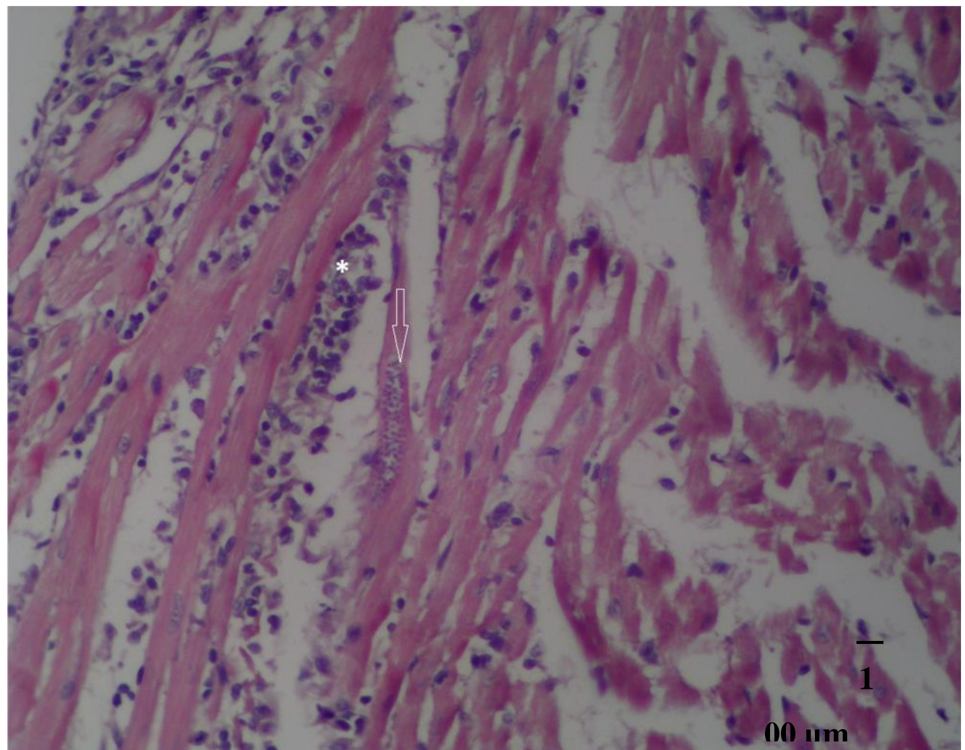


Figure 3: Histopathological image of uninfected mouse heart (A). Cardiac histopathology of mice experimentally infected by *Trypanosoma cruzi* (B) showing pseudocysts containing amastigotes (↓) and inflammatory infiltrate (*).

4.5 MANUSCRITO I

Em revisão na revista **Naunyn-Schmiedeberg's Archives of Pharmacology**

Cordycepin and pentostatin against *Trypanosoma cruzi*

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ABSTRACT

The aim of this study was to evaluate *in vitro* and mice the efficacy of cordycepin and pentostatin (alone or combined) against *Trypanosoma cruzi*. *In vitro*, the cordycepin and pentostatin exerted potent trypanocidal effect against Colombian strain of *T. cruzi*, similarly to benznidazole, which is the reference drug. *In vivo,* the combined use of cordycepin and pentostatin on day one of mice infection reduced parasitemia caused by epimastigotes and trypomastigotes, as well as benznidazole compared to control animals. For epimastigotes, the lethal dose of cordycepin capable of killing 50 % (LD_{50}) and 20 % (LD_{20}) of the parasites was 0.072 mg/mL and 0.031 mg/mL, respectively and for trypomastigotes was 0.047 and 0.015, respectively. The combined use of cordycepin and pentostatin resulted in a LD_{50} and LD20 for epimastigotes of 0.068 mg/mL and 0.027 mg/mL, respectively, as well as 0.056 mg/mL and 0.018 mg/mL for trypomastigotes, respectively. Since the therapeutic protocol did not show an expected curative effect, only reduced the parasitema in the peak period in animals treated with the combination cordycepin and pentostatin. In summary, the combination of cordycepin and pentostatin showed no curative effect in mice infected by *T. cruzi*, despite the *in vitro* reduction of epimastigotes and trypomastigotes.

KEY-WORDS: Chagas disease; epimastigotes; trypomastigotes; 3′-deoxyadenosine; deoxycoformycin.

INTRODUCTION

Chagas disease is a neglected disease caused by the protozoan *Trypanosoma cruzi*, an obligatory intracellular parasite firstly described in 1909 (Bern, 2015; Chagas, 1909). This disease has two clinical phases: acute and chronic where the acute phase lasts approximately 30 days, and the patients have approximately 95 % of survival before reaching the chronic phase. In the chronic phase, 60 % of the individuals remain asymptomatic, a period called the indeterminate phase of chronic Chagas disease and 10 % of these patients develop cardiomyopathies (De Oliveira et al., 2016). According to Oliveira et al. (2015), the treatment of humans against *T. cruzi* is based on nifurtimox and benznidazole. However, collateral effects were reported for nifurtimox (anorexia, abdominal pain, nausea, vomiting and weight loss), as well as for benznidazole (while dermatitis, anorexia, leukopenia and polyneuropathy) (Oliveira et al., 2015). Therefore, there is a need of more effective compounds with less toxicity, such as pentostatin combined with cordycepin to treat individuals infected by *T. cruzi*.

Pentostatin is an inhibitor of adenosine deaminase (ADA) activity (Rottenberg et al., 2005), responsible for the deamination of adenosine into inosine. Thus, an inhibition of ADA activity may enhance ADO levels, which is an anti-inflammatory molecule. In this sense, our hypothesis is that the treatment with pentostatin combined with cordycepin may improve the immune system response through the purinergic system. The therapeutic protocol using the combination of cordycepin and pentostatin has been used successfully in experimental infections caused by *Trypanosoma evansi* and *Trypanosoma brucei* (Rottenberg et al., 2005; Dalla Rosa et al., 2015). Recently, a study conducted by do Carmo et al. (2017) demonstrated the *in vivo* trypanocidal action of cordycepin against trypomastigotes of Y strain of *T. cruzi,* i.e, the treatment reduce parasitemia, but showed no curative effect. However, the trypanocidal action of this treatment against epimastigotes and trypomastigotes of the Colombian strain of *T. cruzi* remains unknown. It should be noted that these compounds are purine analogues, and little is known regarding their side effects on seric levels of purine. The aim of this study was to evaluate the efficacy of cordycepin and pentostatin combined to treat mice infected by Colombian strain of *T. cruzi*.

MATERIAL AND METHODS

Products

Cordycepin® was obtained from Sigma Chemical Co (St. Louis, MO, USA) and pentostatin® from Tocris Bioscience (Minneapolis, MN, USA). Unless otherwise indicated, all reagents were diluted in PBS, aliquoted and stored at –20 ºC until further use. Benznidazole (LAFEPE, Recife, Brazil) was dissolved in dimethyl sulfoxide (DMSO) (Sigma–Aldrich, St. Louis, MO, USA) and used as the reference drug against *T. cruzi*.

Strains

This study used blood containing trypomastigotes and epimastigotes of *T. cruzi*, Colombian strain (Federici et al., 1964) maintained in the Veterinary Parasitology Laboratory of the Universidade Federal de Santa Maria (UFSM), Brazil.

Experiment I: in vitro

The epimastigotes (Colombian strain) of *T. cruzi* were cultured in LIT media (Liver Infusion Tryptose) containing 10 % of fetal bovine serum (FBS), 200 U/mL of penicillin and 100 μg/mL of streptomycin (Gibco, UK) at 28 °C. To obtain the parasite suspension for the assay, the epimastigote culture was concentrated by centrifugation (1000 *x g* for 10 min), and the number of epimastigotes was counted in a Neubauer haemocytometric chamber. The blood trypomastigote forms (Colombian strain) of *T. cruzi* were obtained from an infected mouse at the maximum peak of parasitemia. Blood from this animal was collected by cardiac puncture and stored in tubes with EDTA anticoagulant. The number of parasites was also determined in a Neubauer haemocytometric chamber and the concentration adjusted in LIT medium. The *in vitro* tests against epimastigote and trypomastigote forms of *T. cruzi* were performed as described below: the culture medium containing the parasites (10^4 and 10^5 parasites/mL, respectively) was distributed into microtiter plates (250 μL/well), and mixed with 30 μL of each compound previously diluted in PBS. The compounds tested were cordycepin (0.025, 0.05; 0.1; 0.2 and 0.3 mg/mL) individually; and combined, i.e cordycepin (0.025, 0.05; 0.1; 0.2 and 0.3 mg/mL) + pentostatin (0.01 mg/mL). Benznidazole (20 mg/mL) was used for chemotherapy control. The compounds were dissolved in PBS (cordycepin and pentostatin) and DMSO (benznidazole) and added to the culture media. A control group was used to validate the test composed of PBS. The final DMSO-concentration was 0.01 % in the culture, which is non-

toxic and without growth inhibitory effects on the parasite (data not shown). The effect of each compound against epimastigotes and trypomastigotes forms of *T. cruzi* were evaluated after 24 h, using a Neubauer haemocytometric chamber. The tests were carried out in triplicate.

The results of the *in vitro* tests regarding the trypanocidal effect of both drugs were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test for mean comparison. Differences between groups were rated significant at $P<0.05$. Variables were expressed as mean ± standard errors of the mean (SEM).

The values of LD_{50} and LD_{20} (lethal dose responsible for causing mortality effect on 50 % and 20 % of the parasites, respectively) were estimated using non-linear models in the regression analysis (Gompertz Model and Exponential Model) with a confidence interval of 95 %, through the software Statistica 7.0.

Experiment II: *in vivo*

Thirty female (Swiss) mice with 45 days of age and 20 to 30 g of body weight from the Central Animal House of the Universidade Federal de Santa Maria (UFSM) were used in this experiment. The animals were maintained at a constant temperature (23 ± 1 ºC) on a 12 h of light/dark cycle with free access to food and water. Each mouse was inoculated intraperitoneally with 0.2 mL of blood containing $4x10^3$ trypomastigotes of *T. cruzi* (Colombian strain) obtained from a mouse previously infected. The animals were randomly divided into four groups of six animals each and a control group of uninfected mice was also formed with six animals. The groups were composed as follows: the group A – consisted of uninfected mice; the group B – consisted of infected untreated mice; the group C - formed by infected mice treated on the same day of the infection (day 0) with the combination of cordycepin (2 mg/kg) and pentostatin (0.2 mg/kg); the group D - formed by infected mice treated six days post infection (PI) with the combination of cordycepin (2 mg/kg) and pentostatin (0.2 mg/kg); the group E - formed by infected mice treated six days PI with benznidazole (10 mg/kg). The administration of the drugs occurred for 5 consecutive days (groups C, D and E). The doses of cordycepin and pentostatin used in this study were based on a *T. evansi* study (Dalla Rosa et al., 2015) with successful results. The number of blood trypomastigotes of *T. cruzi* was recorded every 2 days from day 6 up to day 20 PI, and the number of trypanosomes was expressed as parasites/mL.

Statistical analysis

The data were submitted to the normality test, which showed a normal distribution. Then, statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey test. P values <0.05 were considered statistically significant.

RESULTS

Experiment I: *in vitro*

The trypanocidal effect of cordycepin and pentostatin on epimastigotes and trypomastigotes of *T. cruzi* (Colombian strain) is shown in Figure 1. A significant (P<0.001) reduction of live epimastigotes (Figure 1-A) and trypomastigotes (Figure 1-B) was observed in groups treated with cordycepin alone or combined (cordycepin and pentostatin) in all tested doses after 24 hours of the beginning of the test when compared to the control group. The two highest concentrations evaluated killed all parasites after 24h (Figure 1). However, benznidazole was able to eliminate all forms of epimastigotes in cell culture 24 hours after the beginning of the tests, but was unable to kill all trypomastigotes (Figure 1).

The percentage of epimastigotes and trypomastigotes inhibition compared to the control group was, respectively: cordycepin 0.025 mg/mL (18.7 and 34.5 %); cordycepin 0.05 mg/mL (39.4 and 56 %); cordycepin 0.1 mg/mL (67.5 and 83.3 %); cordycepin 0.2 mg/mL (100 % for both forms); cordycepin 0.3 mg/mL (100 % for both forms); cordycepin 0.025 mg/mL + pentostatin 0.01 mg/mL (26.5 and 30 %); cordycepin 0.05 mg/mL + pentostatin 0.01 mg/mL (41.9 and 95.6 %); cordycepin 0.1 mg/mL + pentostatin 0.01 mg/mL (77.2 and 73.7 %); cordycepin 0.2 mg/mL + pentostatin 0.01 mg/mL (100 % for both forms); cordycepin 0.3 mg/mL + pentostatin 0.01 mg/mL (100 % for both forms) and benznidazole (100 and 90 %). The P values for all treatments compared to the control group was < 0.05.

For epimastigotes, the LD_{50} and LD_{20} of cordycepin used alone was 0.072 and 0.031, as well as 0.047 and 0.015 for trypomastigotes, respectively (Table 1). The LD_{50} and LD_{20} for epimastigotes was 0.068 and 0.027, as well as 0.056 and 0.018, respectively for trypomastigotes (Table 1) when the combination of cordycepin and pentostatin was used.

Experiment II: *in vivo*

The trypanocidal effect of cordycepin and pentostatin on trypomastigotes of *T. cruzi* (Colombian strain) is shown in Figure 2. The trypomastigote forms of *T. cruzi* were observed

in the bloodstream 6 days PI, except for the group C that showed trypomastigotes on 10 days PI. The peak of parasitemia occurred between days 10 and 14 PI, differing in the number of trypomastigotes between groups. The group C showed lower number of trypomastigotes in the blood between days 16 and 20 PI, reaching the end of the experiment with a significant reduction ($P<0.05$) when compared to the other groups. During the experiments, the animals showed no apparent clinical signs of the disease.

Macroscopically, no cardiac changes were observed in all mice of the experiment. Microscopic examination revealed the presence of pseudocysts containing amastigotes, associated with mild to moderate multifocal inflammatory infiltrate of macrophages in the heart of mice experimentally infected by *T. cruzi* (the group B) (Fig. 3). However, in the groups treated with the combination of cordycepin and pentostatin (groups C and D) only mild to moderate multifocal inflammatory infiltrate of macrophages were observed. Moreover, animals treated with benznidazole showed inflammatory infiltrate similarly to the others groups, and some animals showed amastigote forms.

DISCUSSION

In this present study, an *in vitro* dose-dependent effect of cordycepin and cordycepin associated with pentostatin against trypomastigotes and epimastigotes forms of *T. cruzi* was observed, similarly as observed by Nakajima-Shimada et al. (1996) against amastigotes of *T. cruzi.* According to these authors, cordycepin at concentrations of 5 and 50 µM were able to reduce the infection rate and the number of amastigotes of *T. cruzi* while infecting HeLa cells, with an inhibitory concentration (IC_{50}) of 5 µM. Similarly, a study conducted by Dalla Rosa et al. (2013) demonstrated a dose-dependent *in vitro* effect of cordycepin (alone or combined with pentostatin) against the trypomastigote form of *T. evansi* at concentrations of 0.5 to 10 mg/mL. Pentostatin is an ADA inhibitor that can prevent degradation of cordycepin, and thus, a combination of these two drugs (cordycepin and pentostatin) has been effective against *T. brucei* (Rottenberg et al., 2005; Vodnala et al., 2009) and *T. evansi* (Dalla Rosa et al., 2013), i.e. improved the trypanocidal action of cordycepin. However, in this study a combination of cordycepin and pentostatin showed similar trypanocidal effects when cordycepin was used alone or combined with pentostatin since the combination of both drugs was unable to potentiate the trypanocidal action of cordycepin against the epimastigote and trypomastigote forms of *T. cruzi,* which is corroborated by their similar lethal doses (LD_{50} and LD_{20}), in disagreement to what was reported by Dalla Rosa et al. (2013). These authors demonstrated that the association of cordycepin and pentostatin reduced 69 % (5 mg/mL) and 81 % (10 mg/mL) the number of

trypomastigotes forms of *T. evansi,* while the same concentrations of cordycepin reduced in 48 % and 55 % the number of trypomastigotes forms, respectively. The exact action mechanism linked with this difference remains unknown, but it might be associated with differences on parasitic resistance (*T. evansi* x *T. cruzi*) and by different forms (epimastigote and amastigote x trypomastigote). In summary, the treatment with cordycepin, alone or combined with pentostatin, can be considered an interesting approach against the epimastigote and trypomastigote forms of *T. cruzi.*

In vivo, we used only the association of cordycepin and pentostatin in different treatments, since previous studies *in vivo* demonstrated that the administration of cordycepin did not result in a complete cure of infection (Aiyedun et al., 1973; Da Silva et al., 2011; Dalla Rosa et al., 2013). *In vivo,* better results regarding the trypanocidal effect was obtained when the combination of pentostatin and cordycepin was used on the day of the infection compared to the other treatments, and it was also able to reduce the number of amastigotes of *T. cruzi* in the heart tissue. The efficacy of cordycepin treatment is related to the protection of this drug against the effect of the enzyme ADA, which is responsible for the deamination of adenosine analogue (Vodnala et al., 2009). In this sense, all protocols were able to reduce the parasitemia during the experimental period, but the treatment on the day of the infection showed better trypanocidal effect, which might be linked to the capacity to inhibit or, at least reduce, the multiplication of the parasite in the bloodstream. Similarly, Dalla Rosa et al. (2013) demonstrated that the treatment with cordycepin and pentostatin (2 and 1 mg/kg, respectively) increased animal longevity and showed 83.3 % of curative effectiveness in mice experimentally infected by *T. evansi,* while the treatment with cordycepin and pentostatin (2 and 2 mg/kg, respectively) inhibited the development of trypomastigote forms of *T. evansi* in the blood with 100 % of curative effectiveness. It is important to emphasize that the association of cordycepin and pentostatin exerted a better *in vivo* trypanocidal action when compared to benznidazole (the reference drug), and also in cases of parasitic resistance to benznidazole (Seguel et al., 2016). In this sense, the association of cordycepin and pentostatin may be considered an interesting alternative to treat infected individuals.

Based on these evidences, the association of cordycepin and pentostatin has trypanocidal action *in vitro*, and it is able to maintain parasitemia at low levels in mice experimentally infected by *T. cruzi*. However, the treatment in the same day of infection had no curative effect, but controlled the blood parasitemia and it reduced cardiac lesions of treated mice.

Ethical Committee

The present study was approved by the Ethics Committee for Use of Animals (CEUA) of the Universidade Federal de Santa Maria (UFSM) under protocol number 8288290615.

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Table 1. Lethal dose capable of killing 50 % of the parasites (LD_{50}) and lethal dose capable of killing 20 % of the parasites (LD_{20}) for cordycepin and the combination cordycepin and pentostatin against epimastigote and trypomastigote forms of *Trypanosoma cruzi*. Results were expressed as mg/mL.

Parasitic form	Treatment	DL_{50} (IC 95 %)	DL_{20} (IC 95 %)
Epimastigotes	Cordycepin	0.072 (0.05 - 0.08)	0.031 (0.02 - 0.04)
	Cordycepin + pentostatin	0.068 (0.05 - 0.06)	0.027 (0.0 - 0.02)
Trypomastigotes	Cordycepin	0.047 (0.039 - 0.055)	0.015 (0.012 - 0.017)
	Cordycepin + pentostatin	0.056 (0.044 - 0.067)	0.018 (0.014 - 0.021)

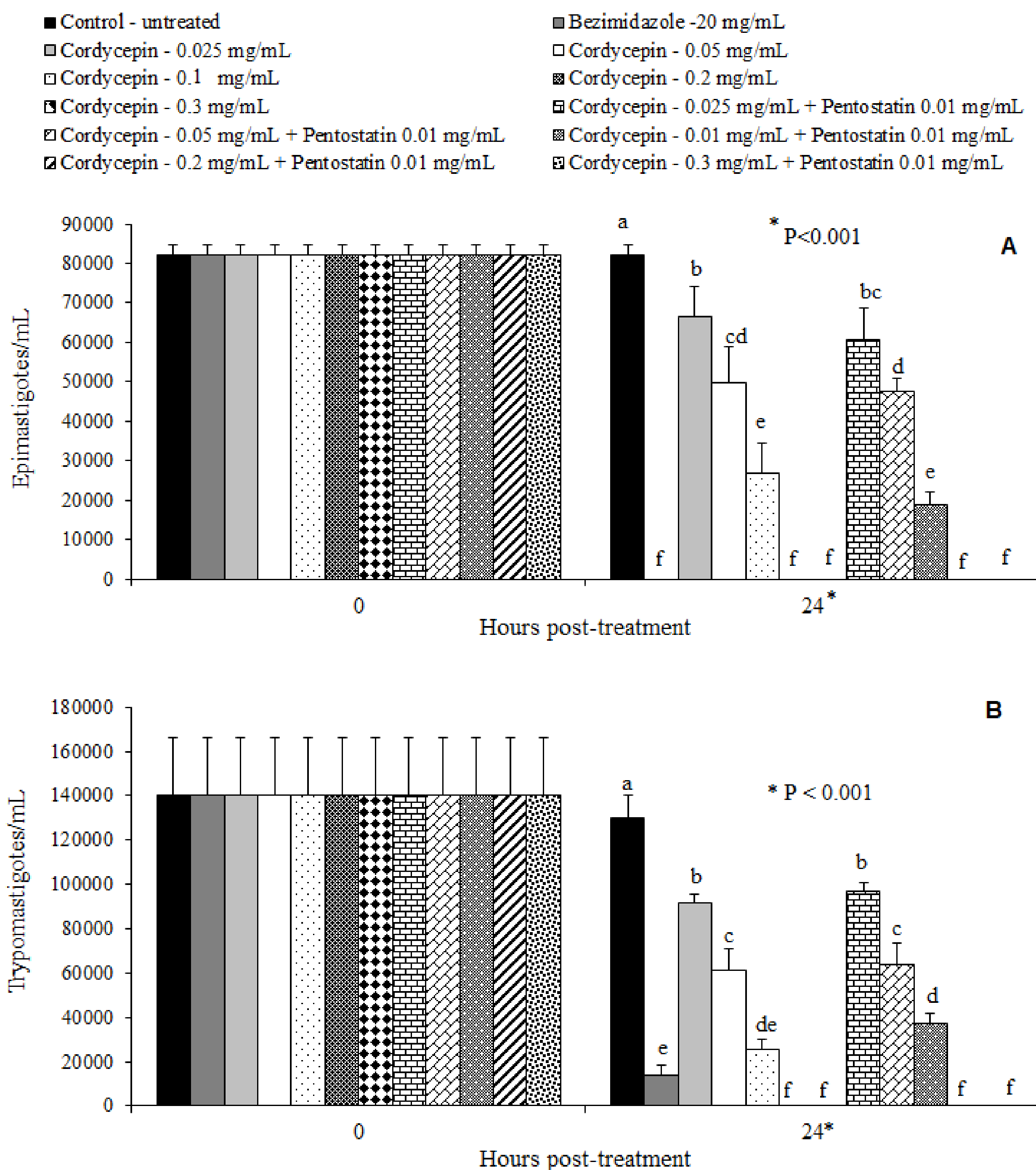


Figure 1. *In vitro* trypanocidal effect of cordycepin and the combination of cordycepin and pentostatin against epimastigotes (A) and trypomastigotes (B) of *Trypanosoma cruzi* (Colombian strain). It was also used a control group composed of uninfected and untreated animals and another group of infected animals treated with benzimidazole (drug conventionally used for treatment). Tukey test (P<0.001 to 24 hours - but equal letters do not differ from each other, by treatment). Results were expressed as mean and standard deviation.

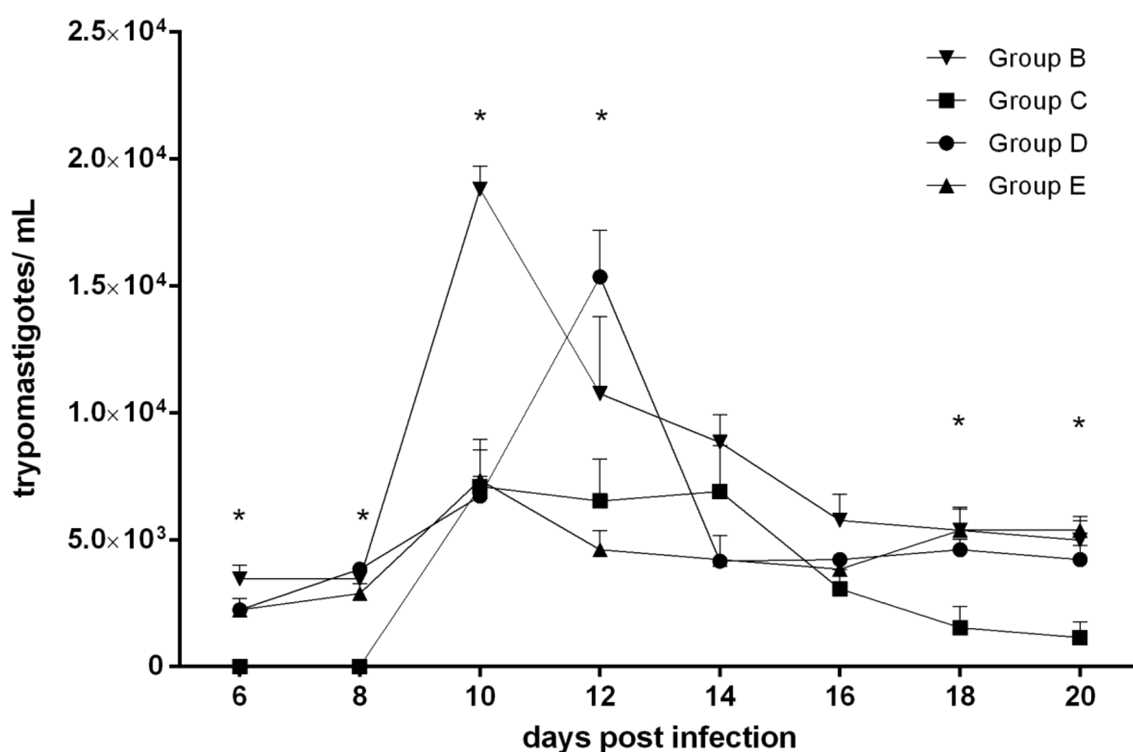


Figure 2. *In vivo* trypanocidal effect of pentostatin and cordycepin used to treat mice experimentally infected by *Trypanosoma cruzi* (Colombian strain). One-way analysis of variance (ANOVA) followed by the Tukey post-hoc analysis (*P<0.05). Note: Infected control (the group B); infected mice treated on the same day of infection with the combination of cordycepin and pentostatin (the group C); Infected mice treated 6 days post infection with the combination of cordycepin and pentostatin (the group D); Infected mice treated 6 days post infection with benznidazole (the group E).

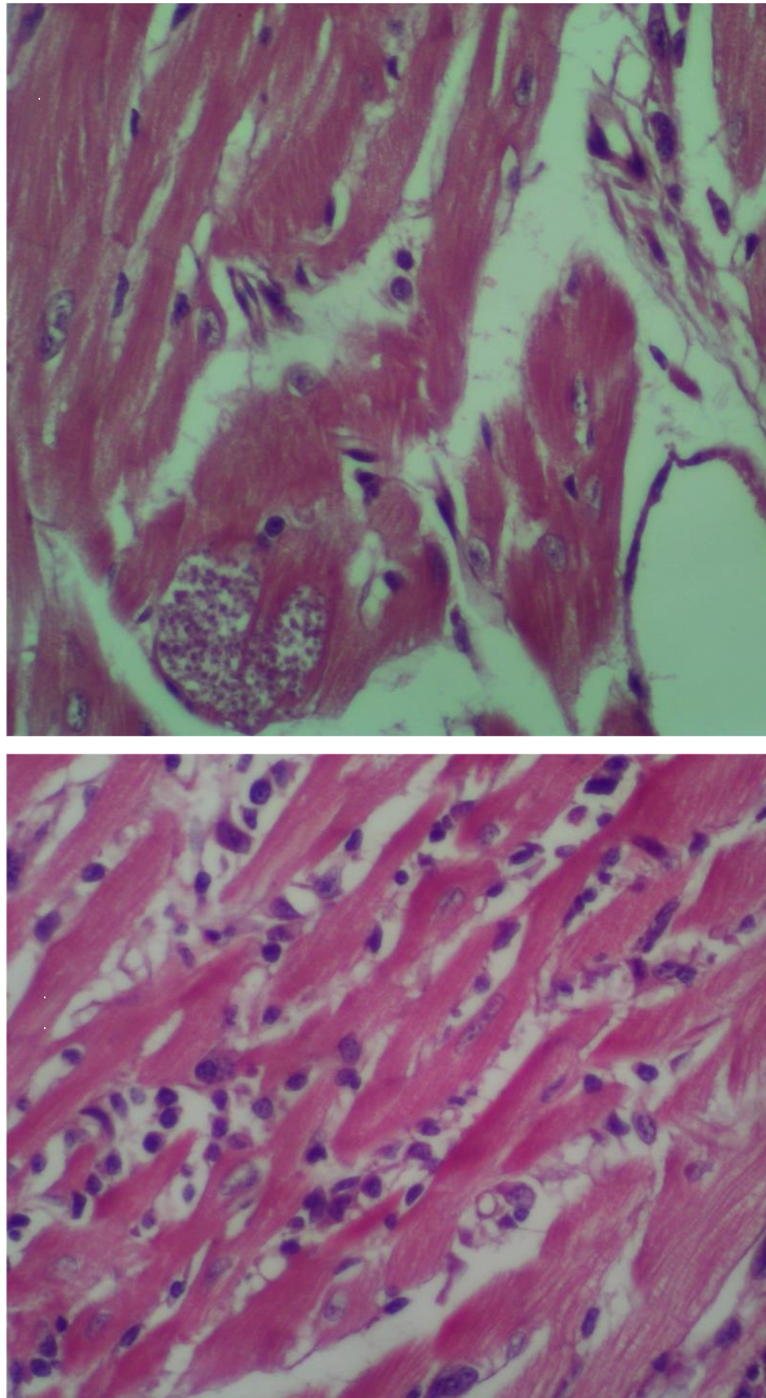
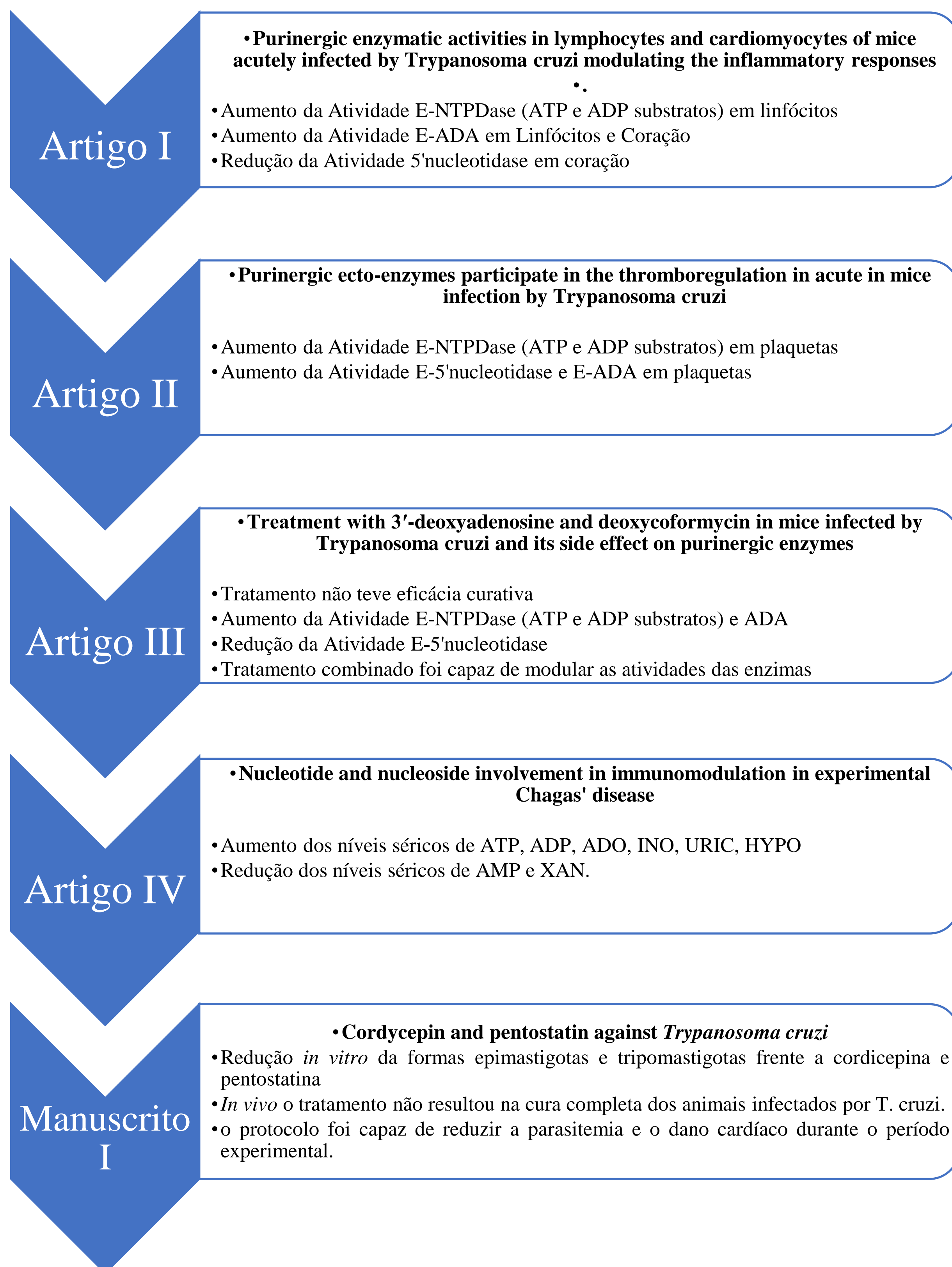


Figure 3. (A) Cardiac histopathology of mice experimentally infected by *Trypanosoma cruzi* showing pseudocysts containing amastigotes (↓) (the group B - infected and untreated). (B) mild to moderate multifocal inflammatory infiltrate of lymphocytes (*) (the group C - infected and treated with cordycepin and pentostatin). HE, Obj 40X.

5 DISCUSSÃO

Os principais resultados desta tese estão elencados no esquema abaixo:



Os resultados obtidos neste trabalho demonstraram que a infecção causada por *T. cruzi* (cepa Y) causou anemia, leucopenia e trombocitopenia nos camundongos, resultados semelhantes a outros encontrados na literatura (Cardoso e Brener 1980; Marcondes et al., 2000). Associadas a estas alterações neste estudo foi observado um aumento na atividade das enzimas do sistema purinérgico em linfócitos e plaquetas de camundongos infectados com *T. cruzi.* O ATP e o ADP são mediadores, liberados para a circulação sanguínea exacerbadamente, em resposta a lesões teciduais e outras condições patológicas, como inflamação, hipoxia e isquemia. A atividade aumentada de E-ADA reduz os níveis de adenosina, molécula esta considerada anti-inflamatória e anti-plaquetária (Anfossi et al., 2002; Bours et al., 2006). Portanto, estas mudanças nas enzimas purinérgicas em linfócito e plaquetas participam da modulação da doença de Chagas, principalmente relacionada ao processo inflamatório e na tromboregulação.

O aumento das atividades de E-ADA em linfócitos, plaquetas e tecido cardíaco pode ser uma resposta fisiológica para reduzir as concentrações de adenosina extracelular. A adenosina possui efeitos anti-inflamatórios (Cordero et al., 2001), também é um importante modulador do tônus vascular e conhecida por inibir a agregação plaquetária. Portanto, a redução da adenosina no meio extracelular aumenta a resposta inflamatória e a agregação plaquetária na tentativa de combater a infecção por *T. cruzi* e evitar hemorragias ou trombocitopenia grave. Assim evitando maiores danos aos tecidos.

Neste trabalho foi observado uma redução significativa na atividade da 5'-nuceotidase no tecido cardíaco e um aumento significativo na atividade da 5'-nuceotidase em plaquetas de camundongos infectados com *T. cruzi* (cepa Y). Esta enzima desempenhou um efeito pró-inflamatótio na infecção aguda por *T. cruzi*, pois esta enzima é responsável pela hidrolise do AMP em adenosina, sendo assim, ocorreu uma redução da adenosina, uma mólecula anti-inflamatória (Junger 2011). Juntamente com o aumento da 5'-nucleotidase em plaquetas, a enzima E-ADA também aumentou, o que demonstra que estas enzimas estão correlacionadas nos mecanismos de tromboregulação da doença de chagas. Alterações de atividades enzimáticas já foram relatadas em muitas doenças na literatura (SCHETINGER et al., 2007; OLIVEIRA et al., 2011; CASTILHOS et al., 2015) e um aumento semelhante também foi verificado na 5'-nuceotidase de plaquetas de uma forma indeterminada da doença de Chagas em humanos (SOUZA et al., 2012).

Considerando que as enzimas do sitemas purinérgico estão envolvidas na fisiopatologia da doença de chagas, como já vistos no artigo I e II desta tese, assim como a cordicepina (análogo da adenosina) possui atividade relatada como de inibição da agregação plaquetária (CHO et al., 2006), inibição da inflamação (ZHANG et al., 2014) e ação tripanocida (VODNALA et al., 2008) e a pentostatina um análogo da desoxiadenosina e um potente inibidor de adenosina deaminase, é importante definir os efeitos do tratamento nas enzimas purinérgicas. Neste estudo, observou-se uma fraca ação tripanocida in vivo de cordicepina contra *T. cruzi* (cepa Y), semelhante ao do benzonidazol. Além disso, a cordicerina e a pentostatina usadas isoladamente ou combinadas, mas principalmente o tratamento associado, conseguiram reduzir o grau de infiltrado inflamatório cardíaco. No entanto, o tratamento com cordicepina e pentostatina (isolado ou associado) não teve êxito para curar os camundongos infectados experimentalmente pelo parasita. Portanto este protocolo terapêutico, na dose utilizada, não deve ser recomendado para tratar a doença de Chagas. Por outro lado, o tratamento foi capaz de modular enzimas purinergicas em camundongos infectados por *T. cruzi*, o que pode contribuir para minimizar os infiltrados inflamatórios cardíacos.

Quando utilizamos a cepa colombiana (cepa resistente ao tratamento com benzonidazol) verificamos que os animais infectados apresentaram concentrações séricas aumentadas de ATP, ADP e ADO, enquanto os níveis de AMP diminuíram, semelhante ao observado na literatura (DA SILVA et al., 2012). Estes resultados podem estar ligados a alterações nas enzimas do sistema purinérgico, tais como NTPDase, 5'-nucleotidase e ADA encontrados nos artigos I e II desta tese. Também se observou um aumento nos níveis séricos de inosina e ácido úrico em animais infectados por *T. cruzi* (cepa colombiana), isto pode ser considerado uma tentativa do organismo de minimizar o dano inflamatório, o que corrobora com os achados histopatológicos. Estudos demonstraram que a inosina, hipoxantina, xantina e ácido úrico possuem potente ação imunomudolatória, antiinflamatória e protetora (SANTOS et al., 1999; HASKO e CRONSTEIN 2004).

Sabendo que os níveis de purinas são alterados na infecção por *T. cruzi* (cepa colombiana) visto no artigo IV desta tese e que a cordicepina e pentostatina modularam as enzimas do sistema purinergico, mostrado no artigo III desta tese, resolvemos testar a eficácia da cordicepina e pentostatina frente a infecção causada pela cepa colombiana. No presente estudo foi observado *in vitro* uma redução das epimastigotas e tripomastogotas frente a cordicepina e pentostatina (isolado ou combinado). A DL50 e a DL20 da cordicepina utilizada foi de 0,072 mg/mL e 0,031 mg/mL frente as formas epimastigotas, bem como 0,047 mg/mL e 0,015 mg/mL para os tripomastigotas, respectivamente. Assim como a DL 50 e a DL20 da

combinação de cordicepina e pentostatina para epimastigotas foi 0,068 mg/mL e 0,027 mg/mL, bem como 0,056 mg/mL e 0,018 mg/mL para os tripomastigotas, respectivamente. Apenas desse potente efeito in vitro, *in vivo* a associação de cordicepina e pentostatina não resultou na cura completa dos animais, porém, os protocolos de tratamento foram capazes de reduzir a parasitemia durante o período experimental e o dano cardíaco.

6 CONCLUSÃO

Em resumo, houve aumento observado na atividade das enzimas do sistema purinérgico em linfócitos e plaquetas de camundongos infectados com *T. cruzi* (cepa Y) estão diretamente ligadas a inflamação e tromboregulação investigados nessa tese. As alterações nessas enzimas estavam diretamente relacionadas a regulação de purinas extracelular, onde destacamos ATP, ADP, adenosina e acido úrico que atuam na modulação da resposta inflamatória (ATP e adenosina), hemostasia (ADP e adenosina), assim como a tentativa de minimizar o dano inflamatório causado pelo parasita (acido úrico). Todas essas alterações compiladas permite a primeira conclusão, isto é, o sistema purinergico esta envolvido diretamente e indiretamente na fisiopatologia e patogenia da Doença de Chagas.

A segunda conclusão é a ineficácia da cordicepina e pentostatina (isolados ou combinados) frente a cepa Y de *T. cruzi* foi observado, pois não demonstraram um efeito curativo nos camundongos. Apesar do tratamento ser capaz de reduzir o dano cardíaco causado pelo parasita e modular as enzimas do sistema purinérgico. Quando foi testado a eficácia do tratamento com cordicepina e pentostatina (isolado ou combinado) frente a cepa resistente ao benzonidazol (cepa colombiana) foi possível concluir que *in vitro*, o tratamento reduziu as formas epimastigotas e tripomastigotas, mas *in vivo*, o tratamento também não resultou na cura completa dos animais, porém, foi capaz de reduzir a parasitemia durante o período experimental. Portanto, o protocolo experimental testado não é uma opção para tratamento de Doença de Chagas individualmente, mas não impede novas pesquisa que podem vir a combinar esse tratamento com quimioterapia convencional, potencializando efeito, e aumentando percentual de cura em pacientes.

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ANEXOS

ANEXO I

Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que o Projeto intitulado "ASSOCIAÇÃO DE CORDICEPINA E PENTOSTATIN NO TRATAMENTO DE CAMUNDONGOS INFECTADOS COM Trypanosoma cruzi", protocolado sob o CEUA nº 8288290615, sob a responsabilidade de **Silvia Gonzalez Monteiro** *e equipe; Guilherme Machado do Carmo; Luciana Dalla Rosa; Mariangela Facco de Sá; Matheus Baldissera Dellaméa* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 10/12/2015.

We certify that the proposal "CORDYCEPIN AND PENTOSTATIN ASSOCIATION TREATMENT IN MICE INFECTED WITH Trypanosoma cruzi", utilizing 162 Heterogenics mice (162 females), protocol number CEUA 8288290615, under the responsibility of **Silvia Gonzalez Monteiro** *and team; Guilherme Machado do Carmo; Luciana Dalla Rosa; Mariangela Facco de Sá; Matheus Baldissera Dellaméa* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 12/10/2015.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 08/2015 a 08/2016 | Área: Microbiologia E Parasitologia

Procedência:	Biotério Central UFSM						
Espécie:	Camundongos heterogênicos	sexo:	Fêmeas	idade:	60 a 60 dias	N:	162
Linhagem:	Swiss			Peso:	25 a 25 g		

Resumo: O agente etiológico da doença de Chagas é o protozoário flagelado chamado de Trypanosoma cruzi, que segundo a Organização Mundial da Saúde cerca de 6-7 milhões de pessoas são acometidas. Sabendo que a terapêutica da doença de Chagas ainda permanece ineficaz este trabalho tem como objetivo avaliar a associação de cordicepina e pentostatin no tratamento de camundongos infectados com Trypanosoma cruzi. Para isto serão utilizados 162 camundongos adultos, fêmeas divididos e 3 grandes grupos (cepa Y, CL e Colombiana) e seis subgrupos (controle positivo, controle negativo, cordicepina, pentostatin, cordicepina + pentostatin e controle farmacológico), destes apenas o controle negativo não serão infectados com Trypanosoma cruzi. A inoculação será realizada por via intraperitonial com 104 formas tripomastigotas das três diferentes cepas de T. cruzi. Haverá dois momentos de coletas para os grupos infectados (15 e 60 dias pós-infecção). Após o tempo determinados estes animais serão anestesiados com isofluorano, assim será coletado o sangue destes animais para as análises bioquímicas e posterior a decapitação será coletado o cérebro e o coração destes, para verificar um possível desiquilíbrio energético e ou oxidativo.

Local do experimento: Biotério de experimentação do LAPAVET, prédio 20 subsolo

Santa Maria, 23 de maio de 2016

Profa. Dra. Daniela Bitencourt Rosa Leal
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Denis Broock Rosemberg
Vice-Coordenador da Comissão de Ética no Uso de Animais
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ANEXO II

Experimental Parasitology 175 (2017) 44–50

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Experimental Parasitology

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Purinergic enzymatic activities in lymphocytes and cardiomyocytes of mice acutely infected by *Trypanosoma cruzi* modulating the inflammatory responses

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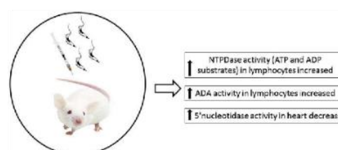
HIGHLIGHTS

- Chagas disease is a zoonosis caused by *Trypanosoma cruzi*.
- Activation of NTPDase can reduce ATP levels, an important inflammatory mediator.
- The ADA also had increased activity; however, by hydrolyzing adenosine, an anti-inflammatory molecule.
- These alterations may implicate in the pathophysiology of Chagas disease related of inflammatory response.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of this study was to evaluate the activity of purinergic enzymes in lymphocytes and cardiac tissue of mice experimentally infected by *Trypanosoma cruzi*. Twelve female mice were used, divided into two groups ($n = 6$): uninfected and infected. On day 12 post-infection (PI), the animals were anesthetized and after euthanized, and samples were collected for analyses. Infected mice showed reduction in erythrocyte counts, hematocrit and hemoglobin concentration, as well as reduced number of total leukocytes in consequence of neutrophilia ($P < 0.01$). The number of monocytes increased in infected mice ($P < 0.001$), however the number of lymphocytes and eosinophils did not differ between groups ($P > 0.05$). The E-NTPDase (ATP and ADP substrate) and E-ADA activities in lymphocytes increased significantly in mice infected by *T. cruzi* ($P < 0.01$). In the heart, multiple pseudocysts containing amastigotes within cardiomyocytes were observed, as well as focally extensive severe necrosis associated with diffuse moderate to severe inflammatory infiltrate of lymphocytes. Although, the NTPDase activity (ATP and ADP substrate) in the cardiac homogenate did not differ between groups, a reduction on 5'-nucleotidase activity ($P < 0.001$) and an increase in the ADA activity in infected animals ($P < 0.05$) were observed. Thus, animals infected by *T. cruzi* experienced the disease, i.e., showed anemia, leucopenia, and

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ANEXO III

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Mol Cell Biochem (2017) 432:1–6
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Purinergic ecto-enzymes participate in the thromboregulation in acute in mice infection by *Trypanosoma cruzi*

Guilherme M. do Carmo[1] · Pedro H. Doleski[2] · Mariângela F. de Sá[2] · Thirssa H. Grando[2] · Nathieli B. Bottari[1] · Daniela B. R. Leal[2] · Lucas T. Gressler[2] · Ricardo E. Mendes[3] · Lenita M. Stefani[4] · Silvia G. Monteiro[2] · Aleksandro S. Da Silva[1,4]

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Abstract Coagulation disorders have been described in Chagas disease with thrombocytopenia as an important event. Several mechanisms may be related to this pathogenesis, such as enzymes of the purinergic system, purine, and receptors involved in the regulation and modulation of physiological events related to hemostasis. Therefore, the aim of this study was to evaluate the activities of E-NTPDase, E-5′nucleotidase, and ecto-adenosine deaminase (E-ADA) in platelets of mice experimentally infected by *Trypanosoma cruzi*. Twelve female mice were used, divided into two groups ($n=6$): uninfected and infected. Mice of infected group were intraperitoneally inoculated with 10^4 trypomastigotes of *T. cruzi* (strain Y). On day 12 post-infection (PI), blood samples were collected for quantitation and separation of platelets. A significant reduction in the number of platelets of infected mice ($P<0.05$) was observed. The activities of E-NTPDase (ATP and ADP substrates), E-5′nucleotidase, and E-ADA in platelets increased significantly ($P<0.05$) in mice infected by *T. cruzi* compared with uninfected animals. A negative correlation ($P<0.01$)was observed between the number of platelets and ATP hydrolysis ($r = -0.64$), and ADP hydrolysis ($r = -0.69$) by E-NTPDase. Therefore, there is a response from the purinergic system activating ecto-enzymes in platelets of mice *T. cruzi* infected, as a compensatory effect of thrombocytopenia.

Keywords Chagas disease · Coagulation disorders · Purinergic ecto-enzymes

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Introduction

The involvement of the purinergic system in coagulopathies is known [1, 2]. The purinergic signaling system plays an important regulatory role in cellular activation, blood flow, and vascular thrombosis by extracellular biomolecules, such as adenine nucleotides and their derivative nucleoside adenosines [3]. According to the literature, the adenine nucleotides ATP, ADP, and AMP, and the nucleoside adenosine regulate and modulate many cellular functions, including platelet aggregation [1]. It is important to highlight that the ADP is primarily responsible for promoting this aggregation, while adenosine is a potent inhibitor [4, 5]. Its anti-aggregant effect is mediated via G-protein coupled adenosine receptors (P1 purinoceptores), specifically the A2B adenosine receptor subtypes [6]. Therefore, enzymatic activities of E-NTPDase, E-5′-nucleotidase, and adenosine deaminase (E-ADA) are associated with essential mechanisms for thromboregulation and homeostasis [7], by regulating ATP, ADP, AMP, and adenosine [3]. Reports indicate that purinergic enzymes are associated with thrombocytopenia in trypanosomosis [7], that can also occur in Chagas disease, due to disorders in coagulation events [8].

Chagas disease is caused by *Trypanosoma cruzi*, a parasitic illness that infects the blood and several tissues of the

ANEXO IV

Microbial Pathogenesis 113 (2017) 51–56

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Microbial Pathogenesis

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Treatment with 3′-deoxyadenosine and deoxycoformycin in mice infected by *Trypanosoma cruzi* and its side effect on purinergic enzymes

Guilherme M. do Carmo[a], Pedro H. Doleski[b], Mariângela F. de Sá[b], Thirssa H. Grando[b], Maria I. Azevedo[b], Alessandra G. Manzoni[b], Daniela B.R. Leal[b], Lucas T. Gressler[b], Luan C. Henker[c], Ricardo E. Mendes[c], Matheus D. Baldissera[b], Silvia G. Monteiro[b], Lenita M. Stefani[d], Aleksandro S. Da Silva[a,d,*]

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Cordycepin
Pentostatin
Purinergic enzymes
Pathogenesis

ABSTRACT

The aim of this study was to evaluate the efficacy of 3′-deoxyadenosine and deoxycoformycin combination in the treatment of mice infected by *T. cruzi*, as well as to verify the influence of the treatment on purinergic enzymes. Heart and serum samples were collected from 60 mice (30 infected and 30 uninfected) at day 12 post-infection. To verify treatment efficacy, parasitemia was monitored, and the treatment with 3′-deoxy adenosine and deoxycoformycin combination was able to reduce it, but had no curative effect on mice. Seric activities of NTPDase (ATP and ADP substrate) and ADA were increased significantly in untreated mice infected by *T. cruzi* compared to the negative control, as well as mice treated with 3′-deoxyadenosine and deoxycoformycin (alone or combined) modulated the activity of NTPDase (ATP and ADP substrate), preventing them from increasing in infected animals (activity similar to healthy animals). Treatment with deoxycoformycin alone and associated with 3′-deoxyadenosine modulated the activity of ADA preventing them from increasing in infected animals. However, seric activities of ADA in mice treated with 3′-deoxyadenosine (cordycepin) alone does not modify the ADA activity compared with infected and non-treated mice. However, the 5′-nucleotidase activity decreased significantly in infected untreated animals and the same occurred in infected and treated animals with deoxycoformycin and 3′-deoxyadenosine. However, treatment with deoxycoformycin associated with 3′-deoxyadenosine preventing them from decreasing the 5′-nucleotidase activity. Therefore, we conclude that the treatments did not have curative success for mice infected by *T. cruzi*. However, the treatments were able to modulate the purinergic enzymes during the infection by *T. cruzi*, which may contribute to reduce the inflammatory damage in heart.

1. Introduction

Chagas disease is a zoonosis caused by the protozoan *Trypanosoma cruzi* and was first described in 1909 [1]. This is a neglected disease in the world and remains a serious health problem with approximately 8–10 million people infected in the world [2]. The main drugs used for the treatment of Chagas disease are benznidazole and nifurtimox, despite their significant side effects [3]. In Brazil, benznidazole is available to treat patients with approximately 80% of effectiveness in the acute phase of the disease, and < 20% of the patients with chronic Chagas disease [4,5].

An analogue product of purine, 3′-deoxyadenosine (cordycepin), was effective to cure mice infected by *Trypanosoma brucei* in both the acute and chronic phases (with central nervous system involvement) of the disease [6–8]. Another study tested the same treatment and the same dose in mice infected by *Trypanosoma evansi*, and obtained 100% curative efficacy [9,10]. Besides that, the biological effect of cordycepin against *T. brucei*, *T. cruzi*, and *Leishmania* sp. was also observed *in vitro* [6]. According to the literature, this effect is due to an inability of trypanosomes to engage in new purine synthesis, which has been exploited as a therapeutic target [11].

The cordycepin combined with pentostatin has been used to treat

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ANEXO V

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Nucleotide and nucleoside involvement in immunomodulation in experimental Chagas disease

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Abstract
The aim of this study was to evaluate whether *Trypanosma cruzi* infections cause alterations in the levels of seric purines, which could contribute to host immunomodulation. Twelve mice were divided into two groups identified as control (uninfected) and infected (*T. cruzi*) groups. The influence of the disease on seric purine levels was verified on day 20 post-infection (PI) by HPLC. Infected mice had circulating trypomastigotes during the experiment, as well as amastigote forms in the heart associated with inflammatory infiltrates. Increases on adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine (ADO), inosine (INO), and uric acid (URIC) levels were observed in the infected animals, while the adenosine monophosphate (AMP) and xanthine (XAN) levels were reduced compared with mice of the control group, indicating a possible impairment on the purinergic system, and consequently, on the immune system during the clinical course of the disease. In summary, the *T. cruzi* infection alters the seric purine levels, and consequently, modulates the immune system.

Keywords Chagas disease · Nucleosides · Nucleotides · Purines · Inflammatory response

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Introduction

Trypanosoma cruzi is the etiologic agent of Chagas disease, which is generally transmitted to humans through the fecal droppings of the infected triatomine insects belonging to the Reduviidae family, being considered an important public health problem in Latin America, since the infection is endemic in this region [1]. Recently, the World Health Organization estimated that approximately seven million people are currently infected by *T. cruzi* in the endemic regions, with a risk to develop the chronic form of the disease [2]. Although the transmission by insects is considered the most important source of infection, other mechanisms of transmission are also known, such as blood transfusions, congenital, and ingestion of contaminated sugarcane juice and açaí palm fruit, and organs fµor transplants [1, 3].

In humans, heart diseases are the main consequence of Chagas disease; however, other tissues and systems can be affected. Despite the wide variety of studies, the mechanisms are not yet fully understood as to how the parasite invades mammalian host cells. Recently, it was verified that acute mice infections caused by *T. cruzi* led to the involvement of enzymes of the purinergic system on the pathogenesis of the

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